I hereby certify that I have been publicly appointed and sworn in by the President of the Regional Court (Landgericht) No. I in Munich, Germany, as a translator for the English language. As such I further certify that the following text is a true and complete translation from German into English of a Patent Application filed by BOEHRINGER INGELHEIM INTERNATIONAL GMBH with the German Patent Office on April 17, 1989, Application No. P 39 13 101.7.

So declared in Munich,
Federal Republic of Germany,
this 2nd day of August 1996

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TNF-α binding proteins and DNAs coding therefor

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The present invention relates to DNA, in particular to recombinant DNA, for preparing proteins with the ability to bind TNF-α.

Tumor necrosis factor (TNF-α) was found for the first time in the serum of mice and rabbits which had been infected with Bacillus Calmette-Guerin and had endotoxins injected thereinto, and was recognized because of its cytotoxic and antitumor characteristics (1). It is above all produced by activated macrophages and monocytes. Numerous cell types which are the targets for TNF have surface receptors with a high affinity for this polypeptide (2); lymphotoxin (TNF-ß) binds to the same receptor (3, 4). TNF-α is identical with a factor designated as cachectin (5) which suppresses lipoprotein lipase and leads to hypertriglyceridemia in the case of chronic inflammatory and malign diseases (6, 7). TNF-α takes probably part in the regulation of growth and in the differentiation and function of cells which play a role in inflammations, immune processes and hematopoiesis.

TNF can exert a positive effect on the host organism by stimulation of neutrophils (8, 9) and monocytes and by inhibition of the replication of viruses (10, 11). Moreover, TNF-α activates the immune defense against parasites and acts directly and/or indirectly as a mediator in immunoreactions, inflammatory processes and other processes in the organism, the mode of action being not yet clear in many instances.

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The administration of TNF-α (12) may, however, be accompanied by harmful phenomena (13), such as shock and tissue damage which can be counteracted by antibodies against TNF-α (14). A number of observations suggest that endogenously released TNF-α plays a role in various pathologic states. For instance, TNF-α seems to be a mediator of cachexia which arises in chronic-invasive, for instance parasitary diseases. TNF-α also seems to play an important role in the pathogenesis of shocks caused by Gram-negative bacteria (endotoxin shock); it might take part in some, though not all, effects of lipopolysaccharides (22). Likewise, it has been claimed that TNF plays some part in tissue damage occurring within the frame of inflammatory processes in joints and other tissues and in the lethality and morbidity of the graft-versus-host reaction (GHVR, graft rejection (15). Moreover, a connection between the concentration of TNF in the serum and the deadly end of meningococcal diseases has been reported on (16).

Further, it has been found that the administration of TNF-α over a long period of time causes a state of anorexia and tabes with similar symptoms as cachexia which is accompanied by neoplastic and chronic infectious diseases (23).

There have been reports on a TNF-inhibiting activity of a protein from the urine of febrile patients whose effect is supposed to be due to a competitive mechanism at the receptor plane itself (24) (similar to the effect of the interleukin-1 inhibitor (17)).

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A protein that inhibits the biological effects of TNF-α by preventing the binding of TNF-α to its cell surface receptor through interaction with TNF-α could be identified in the dialysis urine of uremia patients in preliminary tests regarding the present invention.

It was found that the TNF-α binding protein has also an affinity to TNF-ß; this affinity was determined to amount to about 1/50 of the affinity to TNF-α.

This protein, hereinafter called TNF-α binding protein or TNF-BP, was further purified until homogeneity. It has a molecular weight of about 30,000, determined by means of SDS-PAGE, the N-terminal amino acid sequence was determined as
Asp-Ser-Val-X-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-.(In addition, the following N-terminal sequence was detected in traces:
Leu-(Val)-(Pro)-(His)-Leu-Gly-X-Arg-Glu-.)

The native TNF-BP was detected as glycoprotein.

The following amino acid composition was determined, indicated in amino acid residues per molecule and determined as the mean value of a hydrolysis performed for 24 and 48 hours:

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	Mol amino acid/ mol protein	mol % amino acid
Asp + Asn	27.5	10.9
Thr	15.8	6.3
Ser	20.7	8.2
Glu + Gln	35.0	13.8
Pro	9.5	3.8
Gly	16.0	6.3
Ala	4.2	1.7
Cys	32.3	12.8
Val	10.8	4.3
Met	1.1	0.4
Ile	7.0	2.8
Leu	20.2	8.0
Tyr	6.1	2.4
Phe	8.1	3.2
His	11.1	4.4
Lys	15.7	6.2
Arg	11.8	4.7
Total	252.9	100

A content of glucosamine was detected by means of amino acid analysis.

TNF-BP forms a complex together with TNF-α; the molar ratio of TNF-α to TNF-BP was determined to be 1:1. TNF-BP could be detected in the serum, but not in the urine of healthy persons. An increased titer was detected in the serum and in the urine of dialysis patients with uremia.

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Hence, the urine of uremia patients who are under constant dialysis treatment (hereinafter referred to as dialysis urine or urine from dialysis patients) is especially suited as a starting material for the purification of TNF-α binding protein.

The performed binding tests revealed a dose-dependent inhibition of the TNF binding to the cell by concentrated dialysis urine. The possible interpretation that the observed decrease in binding might be caused by TNF-α itself, which possibly exists in the urine, or by TNF-ß which competes for the binding was ruled out by the finding that the decrease in binding could not be eliminated by the use of TNF-α and TNF-ß antibodies. It was also shown that the effect of the inhibitor is not due to the inhibitor being bound to the cell; rather, proof could directly be furnished that a complex is formed between TNF-α and TNF-BP. Hence, TNF-BP differs in its action from the interleukin-1 inhibitor which competes with interleukin-1 for the binding to the cell surface receptor (17). The interleukin-1 inhibitor does not impair the effect of TNF-α. It was also demonstrated that the TNF-α binding protein shows affinity to TNF-ß.

The TNF-binding protein can be purified, possibly after the dialysis urine has been concentrated, in several steps in accordance with standard protein-chemical methods, e.g. by means of fractionated ammonium sulfate precipitation, ion exchange or gel permeation chromatography, adsorption, e.g. on hydroxyl apatite, or hydrophobic adsorption, affinity chromatography, reversed phase chromatography, wherein the steps can normally be

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taken in any desired order, with a dialysis or gel filtration step being possibly interposed.

A prepurification which was performed in two steps by means of ion exchange chromatography with a DEAE column and subsequent gel chromatography (Sephadex G-75) yielded a 62-fold enrichment.
The purification is preferably continued by affinity chromatography by TNF-α bound to solid carrier material (such as Sepharose); if necessary, a final purification is performed in further chromatographic steps, such as reversed phase chromatography, until homogeneity.

The homogenous protein was obtained in highly purified form by concentrating the urine of dialysis patients by way of ultrafiltration, dialyzing the concentrated urine and enriching it first to the four-fold amount in a fist purification step by means of DEAE Sephacel chromatography. Further enrichment took place by means of affinity chromatography by TNF-α bound to Sepharose. The final purification step was performed by means of reversed phase chromatography (FPLC).

As regards the highly purified protein, the N-terminal amino acid sequence was elucidated.

The occurrence of increased titers of the TNF-binding protein both in the serum and in the urine of uremia patients can be explained by the fact that proteins of such a size which are normally subjected to glomerular filtration and are catabolized by tubule cells are accumulated in the blood and urine in case of diseases

with loss of the nephron (e.g. glomerulonephritis) which are accompanied by a decrease in secretion and endogenous catabolism (18).

TNF-BP has obviously the function of a regulator of the TNF activity with the ability to buffer the changes in concentration of free, biologically active TNF-α. TNF-BP might also influence the secretion of TNF by the kidney because the complex formed with TNF, which has a molecular weight of about 75,000, determined by means of gel permeation chromatography on Sephadex G 75, is not retained by the glomerulus, in contrast to TNF.

The TNF-BP represents one of three main protein components from the urine of dialysis patients that show affinity to TNF and jointly elute with TNF-BP from the TNF affinity chromatography column. The two other proteins, however, obviously bind in a manner which does not impair the binding of TNF-α to its cell surface receptor.

This protein might be the soluble part of the TNF receptor because of the results obtained with respect to the biological activity of the TNF-BP

Because of its ability to inhibit the biological activity of TNF-α and TNF-ß, the TNF-binding protein is suited for use at indications in which a reduction of the TNF activity in the organism is recommended.

TNF-BP can be used for the prophylactic and therapeutic treatment of the human or animal body in the case of

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indications in which a detrimental effect of TNF-α takes place. Such diseases are, in particular, inflammatory and infectious and parasitary diseases or shocks in which endogenous TNF-α is released. These also include pathological states which may occur as side effects in the treatment with TNF-α, especially in case of high dosage, e.g. serious hypotension or disturbance of the central nerve system.
Especially pharmaceutical preparations are suited as drugs for parenteral applications, for instance in the form of lyophilisates or solutions, optionally together with physiologically compatible additives, such as stabilizers. Because of its TNF-α binding properties, TNF-BP is also suited as a diagnostic agent for the determination of TNF-α, e.g. as one of the components in radioimmunoassays or enzyme immunoassays, optionally together with antibodies against TNF-α.

Because of its properties, this protein is a pharmacologically valuable active substance which being from natural sources cannot be represented in sufficient amounts by means of protein-chemical methods.

There has therefore been a need to produce this protein (or related proteins with the ability to bind TNF-α) in a recominbant manner to provide it in sufficient amounts for therapeutic applications.

It has been the object of the present invention to provide DNAs coding for proteins with the ability to bind TNF-α so as to permit, on the basis thereof, the preparation of recombinant DNA molecules with the aid of

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which suitable host organisms can be transformed for producing proteins with TNF-BP activity.

This object has been achieved according to the invention in that hybridizing probes were made on the basis of the N-terminal amino acid sequence and amino acid sequences of tryptic peptides which had been obtained from highly purified TNF-BP and that a cDNA which represents part of the DNA coding for TNF-BP was obtained with the aid of these probes from a suitable cDNA library.

Such a DNA hybridizes with DNAs (or RNAs) coding for TNF-BP or related proteins with the ability to bind TNF-α, or for proteins whose processing yields TNF-BP or TNF-BP related proteins. (For the sake of simplicity, these DNAs (or RNAs) shall hereinafter be called "TNF-BP-DNAs" or "TNF-BP-RNAs"). Processing means the in vivo cleavage of partial sequences of a protein. As far as the N-terminus is concerned, this may be the cleavage of a signal sequence and/or other sequences, and optionally also, when the protein is e.g. the extracellular soluble part of a receptor which projects with the N-terminal segment from the cell membrane, the cleavage of the part of the protein which forms the transmembranic and cytoplasmatic region of the receptor, as far as the C-terminus is concerned.

TNF-BP-DNAs (and TNF-BP-RNAs) also comprise cDNAs derived from mRNAs obtained by alternative splicing (or these mRNAs themselves). "Alternative splicing" means the removal of introns where different splice acceptor and/or splice donor sites are used from the same mRNA precursor.

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The resultant mRNAs differ from one another by the total or partial presence or absence of specific exon sequences, with a possible reading frame shift.

The object of the present invention is therefore the DNA defined in claim 1, including all variants thereof that are suited for hybridizing with TNF-BP-DNAs or TNF-BP-RNAs.

Said variants comprise, e.g., those DNA molecules that are obtained by PCR amplification with the aid of primers whose nucleotide sequence does not exactly correspond to the sequence searched for, e.g. because of restriction sites intended for cloning purposes or because of amino acids not unambiguously determined in the amino acid sequence analysis.

The DNA molecules of the invention can be used for obtaining cDNA clones containing TNF-BP-DNAs from cDNA libraries. Furthermore, the DNAs of the invention can be used as hybridizing probes for mRNA preparations to isolate TNF-BP-RNAs and to prepare therefrom e.g. enriched cNDA libraries which permit a considerably simplified and more efficient screening. Another application is the isolation of the desired DNAs from genomic DNA libraries with the aid of the DNAs of the invention as hybridizing probes.

The object of the invention are also TNF-BP-DNAs which hybridize with the above-mentioned DNA molecules, including their degenerate variants as well as modified

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TNF-BP-DNAs coding for proteins with the ability to bind TNF-α.

The object of the invention are also recombinant DNA molecules containing such DNA sequences.

Such recombinant DNA molecules are suited for cloning DNA coding for proteins with the ability to bind TNF-α, and for the expression thereof in corresponding host organisms.

The object of the invention are also polypeptides which are encoded by such DNAs.

Because of their ability to bind TNF-α, these polypeptides are suited for use in the prophylactic or therapeutic treatment of the human or aminal body at indications where a detrimental effect of TNF-α is observed.

Since a TNF-ß inhibiting effect was also detected in TNF-BP, it (or the related polypeptides), being suitably metered and, optionally, in a form modified with resepct to an enhanced affinity to TNF-ß, can also be used for inhibiting the effect of TNF-ß in organisms.

Furthermore, the object of the invention are therefore pharmaceutical preparations containing an amount of TNF-BP effectively inhibiting the biological activity of TNF-α and/or TNF-ß, or of a related polypeptide with the ability to bind TNF.

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The expression "modified DNAs" stands for such DNAs that are e.g. obtained by mutation, transposition or addition or by shortening TNF-BP-DNAs if these modified sequences code for proteins with the ability to bind TNF-α.

Within the scope of the present invention "ability to bind TNF-α" refers to the characteristic of a protein to bind to TNF-α in such a manner that the binding of TNF-α to the functional part of the receptor is prevented and the effect of TNF-α in human or aminmal organisms is inhibited or offset. This definition includes the ability of a protein to bind also to other proteins, such as TNF-ß, and to inhibit the effect thereof.

Like with possible modifications of the DNA sequence, host organisms suited for the expression are selected, particularly with respect to said biological activity; moroever, the standard critera applicable in the production of recombinant proteins, e.g. compatibility with the selected vector, processing capacity, isolation of the protein, expression characteristics, safety and cost aspects play also a role in the decision on the host organism. The selection of a suitable vector follows from the host intended for the transformation. As a rule, all vectors are suited that replicate and express the TNF-BP-DNAs according to the invention.

As far as TNF-BP-DNA is concerned, and with respect to the expression thereof, it is especially the possible relevance of the two criteria determined in the natural protein, namely glycosylation (see preliminary test 12) and high amount of cysteine residues (see preliminary

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test 9), that must be paid attention to as to the property to bind TNF-α. Therefore, eukaryotes, especially suitable expression systems of higher eukaryotes, are expediently used for the expression.

The screening of cDNA libraries with the aid of hybridizing probes that are derived from amino acid sequences of short peptides sometimes encounters major difficulties because of the degeneration of the genetic code. In addition, this procedure is rendered more difficult if one does not know with respect to a protein, such as TNF-BP, in which tissues it is synthetized. In case of failure of such a method it might then not be possible to ascertain for sure whether this is due to the selection of an unsuitable cDNA library or to the inadequate specificity of the hybridizing probes.

To solve such a problem, the following procedure was adopted according to the invention: A library of the fibrosarcoma cell line HS913 T which had been induced with TNF-α and was present in λ gt11 was used as the cDNA library.

To obtain λ DNA with TNF-BP sequences from this library, the great sensitivity of the polymerase chain reaction (PCR) had been exploited. With the aid of this method it is possible to obtain from a whole cDNA library an unknown DNA sequence which is flanked by oligonucleotides designed on the basis of known partial amino acid sequences and used as hybridizing probes. Such a longer DNA fragment can then be used as a hybridizing probe, for

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instance for isolating cDNA clones, especially the original cDNA clone.

The "Polymerase Chain Reaction" (PCR) is a method to amplify DNA enzymatically in vitro. The method is based on the repetition of a cycle of the three steps: heat denaturation of the DNA, binding of oligodeoxynucleotide primers to sequences complementary thereto and extension of the primers with DNA polymerase, which is successively repeated several times under controlled temperature conditions. Two oligodeoxynucleotide primers which flank the DNA segment to be amplified are used for the PCR amplification of DNA. These oligodeoxynucleotides are designed such that they bind to the opposite DNA strands and are oriented such that the DNA synthesis by the polymerase takes place via the region interposed between the primers. This doubles the amount of said DNA segment in each cycle because the extended products are also complementary to one of the primers and are thus enabled to bind the same. This leads to an exponential accumulation of the specific DNA fragment flanked by the primers. The use of a thermostable DNA polymerase from the bacterium Thermus aquaticus permits the automatization of the PCR. Since this enzyme maintains its activity even after a long incubation at a high temperature (95°C), which is necessary for the denaturation of the DNA, it suffices to add the enzyme once for performing many PCR cycles (31).

The above problem was solved in detail as follows:

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As for the highly purified TNF-α binding protein, the N-terminal amino acid sequence and the amino acid sequences of peptides as obtained by tryptic digestion of the protein were determined.

From these sequences, and with respect to the use thereof in the PCR for the preparation of oligonucleotides, regions were selected, on the one hand, from the N-terminus and, on the other hand, from a tryptic peptide in such a manner that the complexity of mixed oligonucleotides is as low as possible as for hybridization with cDNA. Based on these two regions, a respective set of mixed oligonucleotides were prepared, the one derived from the region at the N-teminus being synthetized in accordance with the mRNA and the one derived from the tryptic peptide being synthetized in a reversely complementary manner as to the mRNA. To faciliate subsequent cloning of a PCR amplified segment, the set of oligonucleotides derived from the tryptic peptide was provided with a BamHI restriction site. λ DNA was then isolated from the TNF-α induced fibrosarcoma cDNA library and a TNF-BP sequence was amplified therefrom by means of PCR. The resultant fragment was cloned and sequenced; it has 158 nucleotides and contains the sequence coding for the tryptic peptide 20 between the two sequence segments derived from the primer oligonucleotides.

This DNA fragment may subsequently be radioactively labeled and used as a probe, e.g. for isolating cDNA clones from the fibrosarcoma library. To this end one may proceed such that plaques are first hybridized with the

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probe, phages of hybridized plaques are isolated and λ DNA is obtained therefrom.

Thereupon, it is possible to prepare single-strand DNA by means of PCR and to directly sequence the same. (This method can be employed for rapidly obtaining sequence information on inserts of recombinant λ clones without the need for adopting the standard method of subcloning DNA fragments in M13 or similar vectors.)

The invention shall now be explained in more detail with reference to the following preliminary tests and examples:

Preliminary Test 1

Competition Binding Test

The presence of TNF-BP was checked by competition with the binding of radioactively labeled recombinant TNF-α (^{125}I-TNF-α) on HL-60 cells. The two-phase method according to Tejedor and Ballesta (19) was used for radioactive labeling with ^{125}I-TNF-α. To this end 50 µl of borate buffer (50 mM, pH 8.4), 10 µl KI (0.125mM in free borate buffer) and 1 mCi 125 I (Amersham) were mixed with 1 µg of recombinant TNF-α (prepared by Genentech, containing $36x10^6$ E/mg, corresponding to 646 E/pmole), containing 0.05% Tween 10. On the assumption that labeled TNF-α does not differ from unlabeled one as regards the binding characteristics, the specific activity was determined by competitive testing.

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A subclone of HL-60 cells (HL-60-10) (20, 21) was used for performing the competition binding test.

The cells were grown in suspension culture in RPMI-1640 medium with 10% of fetal bovine serum (FBS). Cells from the logarithmic growth phase ($7.5x10^6$) were washed with binding buffer (RPMI-1640, 10% FBS, 20mM Hepes pH 7.4) and incubated with 50 mM ^{125}I-TNF-α at 4°C for two hours by rotation in 1.5 ml Eppendorf centrifuge tubes in a total volume of 300 µl. Centrifugation was subsequently carried out at 8000xg for 10 seconds, the precipitate was suspended with ice-cold binding buffer and washed twice to separate free one from membrane-bound ^{125}I-TNF.

Radioactivity was measured with a gamma counter (LKB 172 Clinigamma). The specific binding was defined as the difference between the total binding and unspecific binding that occurred at the 1000-fold excess of unlabeled TNF-α (the unspecific binding was 5-20% of the total binding). The influence of dialyzed urine on the binding of ^{125}I-TNF-α was subsequently measured.

The binding inhibition, caused by various volumes of a concentrated dialysis urine (the used original urine was preserved with EDTA (10 g/l), TRIS (6 g/l), NaN_3 (1 g/l), benzamidine hydrochloride (1 g/l), dialyzed against 0.15 M NaCl, 5mM Hepes, pH 7.4 and 1mM benzamidine hydrochloride and stored at -20°C), was used for constructing a standard curve, the urine volume being plotted against the ratio of bound ^{125}I-TNF-α to maximally bound ^{125}I-TNF-α (B/Bmax). One unit of TNF-

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binding protein was defined as the amount of TNF-BP that reduces the ratio of B/Bmax to 0.5.

To prevent a decrease in the binding of ^{125}I-TNF-α to the cells by TNF-α or TNF-ß, which possibly exists in the sample, these had to be removed or blocked.

To this end antisera were used that had been produced by immunizing rabbits with TNF-α or TNF-ß. The immunoglobulin of 1 µl of TNF-α antiserum was adsorbed on 25 mg protein A Sepharose (Pharmacia) in 200 µl binding buffer at 37°C for one hour. To remove TNF-α possibly existing in the biological samples, these were incubated with the anti-TNF-α bound to protein A Sepharose at 4°C for 12 hours in a volume of 750 µl under rotation. After subsequent centrifugation at 10000xg for 5 minutes the supernatant was used for the competition binding test.

It could be shown in a comparative test that with such a method competition can entirely be eliminated by 10nM exogenous recombinant TNF-α.

To this end various concentrations of TNF-α (1-100nM) were incubated in 7 µl of binding buffer with 1 µl of anti-TNF-α, adsorbed on 25 mg protein A Sepharose, at 4°C for 12 hours under rotation. After centrifugation the supernatants were subjected to the competition binding test. The results of this comparative test are shown in Fig. 2a:

The values, obtained by preincubation with anti-TNF-α

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(●---●), were compared with the corresponding values for TNF-α, which had not been preincubated (o---o). The results show that the decrease in the binding of ^{125}I-TNF-α can be eliminated by 10nM TNF-α if TNF-α is preincubated with antibodies.

Since antibodies against TNF-ß did not show any binding to ^{125}I-TNF-α, they could directly be added to the biological sample. 10 µl of antiserum were mixed with the sample, incubated for one hour and the competition assay was subsequently carried out.

It could be shown in a comparative test that the inhibition of the binding caused by 100 nM of exogenous recombinant TNF-ß (prepared by Genentech, containing 230×10^6 E/mg, corresponding to 3.96 E/pmole) could be offset by adding anti-TNF-ß. To this end various amounts of anti-TNF-ß (1.25-20 µl) were added to the comparative samples with a constant TNF-ß concentration. After an incubation for 60 minutes the mixtures were subjected to the competition binding test. Fig. 2b shows that 10 µl of anti-TNF-ß entirely offsets the binding inhibition caused by TNF-ß 100 nM.

The results of the competition binding tests are shown in Fig. 1:

The binding of 50 pM ^{125}I-TNF-α at 4°C in the presence of dialysis urine with a 20-fold concentration is illustrated by the curve ●---● (the bars show the standard error of the arithmetical mean).

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The fact that the measured decrease in the binding of ^{125}I-TNF-α to the cell is not due to the presence of TNF-α or TNF-ß in the sample is shown by the identical curve in samples with a preceding treatment with TNF-α antibodies bound to protein A Sepharose (o---o), or under addition of 10 µl TNF-ß antiserum (□---□).

Preliminary Test 2

This test was carried out to check whether TNF-BP has affinity to TNF-ß. To this end, the procedure was the same as in preliminary test 1, except for the difference that the cells were incubated with 50 pM ^{125}I-TNF-ß. It was found that TNF-BP has an affinity to TNF-ß, the affinity being about 1/50 of its affinity to TNF-α; this means that 50 units of TNF-BP are required for inhibiting the binding of TNF-ß to the cell by 50%.

Preliminary Test 3

Detection of the formation of a complex of TNF-α with TNF-BP from biological liquids.

These tests were performed with a mixture of ^{125}I-TNF-α with dialyzed urine or serum by means of gel chromatography on Sephacryl. To this end mixtures with or without a 250-fold molar excess were prepared from unlabeled TNF-α.

A 500 µl sample was mixed with ^{125}I-TNF-α at 23°C for 10 minutes at a final concentration of 200 pM and applied to a Sephacryl 200 superfine column (1.5x60 cm, Pharmacia),

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which had been equilibrated with 0.15M NaCL, 5mM Hepes pH 7.4. The column was eluted with 0.15M NACl, 5mM Hepes, pH 7.4, at a flow rate of 25 ml/h. Part of the samples were additionally incubated with a 250-fold molar excess of unlabeled TNF-α (50nM) prior to gel chromatography.

Fig. 3A shows the chromatogram of ^{125}I-TNF-α alone (□---□) and a mixture of 200pM ^{125}I/TNF-α with dialyzed urine from a healthy person (●---●).

Fig. 3B shows the elution profiles of mixtures of 200pM ^{125}I-TNF-α with dialyzed urine from a uremia patient in the absence (●---●) and presence (o---o) of a 250-fold excess of unlabeled TNF-α.

FIG. 3C shows the elution profiles of mixtures of 200pM ^{125}I-TNF-α with serum from a healthy person in the absence (●---●) and presence (o---o) of a 250-fold excess of unlabeled TNF-α.

Fig. 3D shows the elution profiles of mixtures of 200pM ^{125}I-TNF-α with serum from a uremia patient in the absence (●---●) and presence (o---o) of a 250-fold excess of unlabeled TNF-α. Arrows signify the void volume V_0 and the elution volumes of bovine serum albumin (I; molecular weight 67,000), ovalbumin (II; 43,000), chymotrypsinogen (III; 25,000) and ribonuclease (IV; 13,700).

Upon use of dialyzed urine from a healthy person ^{125}I-TNF-α eluted as the single peak according to a molecular weight of about 35,000 (FIG. 3A); the use of dialysis urine from a uremia patient, serum from a healthy person

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and serum from a uremia patient resulted in two main peaks, corresponding to molecular weights of about 35,000 (free ^{125}I-TNF-α) and about 75,000 (complex between ^{125}I-TNF-α and TNF-BP).

Preliminary Test 4:

Partial Purification of TNF-BP

TNF-BP was partially purified from several samples of dialysis urine from uremia patients.

a) Concentration:

To this end 300 ml urine each were concentrated by pressure ultrafiltration (Diaflo cells, equipped with UM-10 membranes (Amicon Inc.)) and by subsequent dialysis against 10mM Tris HCl, pH 8.0, 0.3% sodium azide.

b) Ion Exchange Chromatography

To perform ion exchange chromatography a DEAE Sephacel column (1.5x60 cm, Pharmacia) was charged with the sample (15 ml of concentrated dialysis urine containing 192 E/ml TNF-BP). Elusion was performed with 150 ml of an NaCl/10mM Tris/HCl, pH-8.0 gradient, the NaCl concentration ranging from 0 to 0.4 M. The steep gradient used at the end of elution was used for checking whether additional TNF-BP could be eluted at a high ionic strength. The fractions containing the TNF-binding protein were

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combined, lyophilized, resuspended and dialyzed against 0.15 M NaCl, 5mM Hepes pH 7.4. The ion-exchange chromatogram is shown in Fig. 4A (to obtain the values for the absorption, the illustrated values must be multiplied by 0.3).

c) Gel Permeation Chromatography

The protein solution (5 ml, containing 375 E/ml TNF-BP) obtained from step b) was applied to a Sephadex G-75 superfine column (2.6x90 cm, Pharmacia), equilibrated with 0.15 M NaCl, 5mM Hepes, pH 7.4. The column was eluted at a flow rate of 7 ml/hour. The chromatogram is shown in Fig. 4B; the arrows show the void volume (V_0), the elution volumes of bovine serum albumin (I, molecular weight 67,000), ovalbumin (II, 43,000) and chymotrypsinogen (III, 25,000) (to obtain the values for the absorption, the illustrated values must be multiplied by 0.03).

The TNF-BP containing fractions were combined, lyohilized, resuspended, dialyzed against 0.12 M NaCl, 5mM Hepes pH 7.5 and sterilized by filtration. The protein concentration was determined with the aid of the Biorad Protein Microassay and with albumin as standard.

The partial purification yielded a 62-fold enrichment of the TNF-binding protein; the molecular weight was determined to be about 50,000.

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Preliminary Test 5

Inhibition of the biological activity of recombinant TNF by TNF-BP.

Blocking of the growth-inhibiting effect of TNF-α by TNF-BP was measured on HL-60-10 cells in agar culture. 2000 cells were arranged in layers in 1 ml 0.3% agar on 0.5% agar, each time in a growth medium with 10% fetal bovine serum, in 35 mm tissue culture dishes; the top agar layer of the culture dishes contained various concentrations of TNF-α (0-4pM, based on stock solutions containing 80pM or 320pM TNF-α in growth medium) and a uniform concentration of TNF-BP (13E/ml, based on a stock solution containing 100 E/ml in 0.15M NaCl, 5mM Hepes, pH 7.4). The colonies were counted after 10 days.

Fig. 5A shows the blocking of the growth inhibition of recombinant TNF-α by TNF-BP in agar culture: ●---● TNF-α alone; o---o TNF-α with addition of 13 E/ml TNF-BP. TNF-BP alone did not impair cell growth.

The results of tests with different TNF-BP concentrations (0.4-27 E/ml; stock solutions 1000, 250, 62 and 16 E/ml)) at a constant concentration of TNF-α (2 pM; stock solution 80pM) are illustrated in Fig. 5B (bars respectively stand for the standard deviation from the arithmetical mean). The results of these tests show the dose-dependent effect of the TNF-BP on the biological activity of TNF-α.

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Preliminary Test 6

Binding characteristics of cells after pretreatment with TNF-BP

7.5×10^6 HL-60-10 cells were incubated in 300 ml RPMI, 10% FBS, 20mM Hepes pH 7.4 with TNF-BP (14 E/ml) at 37°C for 20 minutes, then washed twice with ice-cold binding buffer. The competition binding test was then performed with ^{125}I-TNF-α, as indicated in Example 1; the control tests were performed with untreated cells.

An average ratio B/Bmax of 0.95 was obtained. This result shows that the effect of the TNF-BP is not due to its possible binding to the cells.

Preliminary Test 7

Preparation of highly purified TNF-BP

a) Concentration of the urine

200 l of dialysis urine from uremia patients, stored in bottles containing EDTA (10 gl/), Tris (6 g/l), NaN3 1 g/l) and benzamidine hydrochloride (1 g/l) and stored at a cool place, were concentrated by ultrafiltration by means of a highly permeable hemocapillary filter having an asymmetrical hollow fiber membrane (FH 88H, Gambro) to 4.2 l with a protein content of 567 g. The concentrate urine was dialyzed against 10mM/l Tris HCl, pH 8. Like in

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the subsequent steps (apart from reversed phase chromatography), 1mM/l benzamidine hydrochloride was added during this process to counteract proteolytic digestion. All of the subsequent purification steps were carried out at 4°C, unless stated otherwise.

b) Ion Exchange Chromatography

This step was performed as described in Preliminary Test 1. To this end DEAE Sephacel columns (2.5 x 40 cm) were charged with samples of concentrated and dialyzed urine containing about 75 g protein each. Elusion was carried out with 800 ml of an NaCl /10mM Tris/HCL pH-8 gradient, the NaCl concentration amounting to 0 to 0.4 M. The fractions of seven columns containing the TNF-BP, having a total protein content of 114 g, were stored at -20°C.

c) Affinity Chromatography

To prepare the TNF Sepharose column, rTNf-α (15 mg) in 0.1 M $NaHCO_3$, 1 M NaCl, pH 9 (coupling buffer) was coupled to 1.5 g cyanogenic bromide-activated Sepharose 4B (Pharmacia). The Sepharose was swollen in 1mM HCl and washed with couling buffer. After addition of rTNF-α the suspension was rotated at room temperature for 2 hours. The excess of CNBr groups was blocked by rotation for one and a half hours with 1M ethanolamine, pH 8. The TNF Sepharose was alternately washed several times in 1M NACl, 0.1 M sodium acetate pH 8 and 1 M NaCl, 0.1 M boric acid pH 4 and subsequently stored in phosphate-buffered saline with 1 mM benzamidine hydrochloride. The fractions obtained from step b) were set to a concentration of 0.2

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M NaCl, 10mM Tris/HCl, pH 8. The TNF Sepharose was packed into a column and washed with 0.2 M NaCl, 10mM Tris HCl, pH 8, and the TNF-BP containing fractions corresponding to about 30 g protein, were applied at a flow rate of 10 ml/h and profusely washed with 0.2 M NaCl, 10mM Tris HCl, pH 8 until an absorption could no longer be detected in the eluate at 280nm. TNF-BP was then eluted with 0.2 M glycine/HCl, pH 2.5.

The curve of the affinity chromatography is shown in Fig. 6: o---o absorption at 280 nm; ●---● TNF-BP, measured in the competition binding test.

TNF-BP containing fractions of 4 separations were combined and lyophilized up to an end concentration of 10 mg/ml after addition of polyethyleneglycol (MW 6000). The lyophilized sample was dissolved in distilled water and dialyzed against distilled water. (The dialzyed sample (4 ml) was stored in a deeply frozen state.)

This purification step led to a further enrichment by about 9000 times in comparison with the preceding step. (The purification degree in this step could not be determined exactly because a certain amount of TNF-α leaked from the column, thereby possibly masking part of the TNF-BP; such a masking is bound to lead to an inaccurate quantitative determination of the activity of the protein). SDS-PAGE (performed as described in Preliminary Test 8) of the TNF-BP containing fractions showed the elution of three main components with molecular weights of 28,000, 30,000 and 50,000 (Fig. 8, trace A).

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d) Reversed Phase Chromatography

An aliquot amount (1 ml) of the fractions obtained from step c) with an addition of 0.1% trifluoroacetic acid was applied to a ProRPC HR 5/10 column (Pharmacia) connected to an FPLC system (Pharmacia). The column was equilibrated with 0.1% trifluoroacetic acid and charged at room temperature with a linear 15 ml gradient of 10 volume percent to 50 volume percent of acetonitrile containing 0.1% trifluoroacetic acid; the flow rate was 0.3 ml/min. Fractions of 0.5 ml were collected and the absorption at 280 as well as the activity of the TNF-α binding protein were determined with the aid of the competition binding test, as indicated in Example 1, with 0.01 µl sample being respectively used. TNF-BP eluted as a single activity peak according to a sharp UV absorption peak. The results of these assays are shown in Fig. 7.

This last purification step yielded an increase in specific activity by about 29 times; the total increase in activity as compared with the starting material (concentrated dialysis urine) amounted to about 1.1×10^6 times.

SDS-PAGE of the reduced and non-reduced sample, carried out as indicated in Preliminary Test 2, pointed to the presence of a single polypeptide with a molecular weight of about 30,000. (Fig. 8, traces B, C).

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Preliminary Test 8

SDS Polacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (25) on flat gels having a length of 18 cm, a width of 16 cm and a thickness of 1.5 mm and 10 pockets by means of an LKB 2001 electrophoresis unit. The protein content of the samples from purification steps c) and d) (Preliminary Test 7) was determined by means of Bio-Rad Protein Assay and calculated from the absorption at 280 nm, with a content of 1 mg TNF-BP/ml being assigned to an absorption of 1.0.

The samples containing about 25 µm protein (from Preliminary Test 7c) and about 5 µg (from 7d) in reduced (ß-mercaptoethanol) and non-reduced form were applied to a 3% concentration gel and a 5% to 20% linear polyacrylamide gradient gel. Electrophoresis was performed at 25mA/gel without cooling. Phosphorylase B (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carboanhydrase (MW 30,000), soybean trypsin inhibitor (MW 20,100) and a-laktalbumin (MW 14,400) were used as molecular weight markers (Pharmacia). The gels were stained with Coomassie blue in 7% acetic acid/40% ethanol and destained in 7% acetic acid/25% ethanol.

The result of the SDS-PAGE is shown in Fig. 8.

Trace A (affinity chromatography): 3 proteins with molecular weights of about 28,000, about 30,000 and about 50,000 were detected.

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Traces B and C (reversed phase chromatography): Electrophoretograms of the highly purified reduced (B) and non-reduced (C) proteins.

The result of the SDS-PAGE shows that TNF-BP consists of a single polypeptide chain having a molecular weight of about 30,000. (The difference between this molecular weight and the molecular weight of about 50,000 as determined by means of gel permeation chromatography on Sephadex G-75 could be explained by the fact that in gel permeation chromatography a higher molecular weight is feigned because of the molecular form of the protein. Another possibility for the explanation of this discrepancy might be the formation of TNF-BP dimers under non-denatured conditions).

Preliminary Test 9

Amino Acid Analysis

The amino acid analysis was performed with the Beckman High Performance Analyzer System 6300 according to standard provisions.

It revealed the following amino acid composition, indicated in mole of amino acid per mole of protein and in mole percent of amino acid, determined as the mean value of a hydrolysis lasting for 24 hours and 48 hours:

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	Mol amino acid/ mol protein	mol % amino acid
Asp + Asn	27.5	10.9
Thr	15.8	6.3
Ser	20.7	8.2
Glu + Gln	35.0	13.8
Pro	9.5	3.8
Gly	16.0	6.3
Ala	4.2	1.7
Cys	32.3	12.8
Val	10.8	4.3
Met	1.1	0.4
Ile	7.0	2.8
Leu	20.2	8.0
Tyr	6.1	2.4
Phe	8.1	3.2
His	11.1	4.4
Lys	15.7	6.2
Arg	11.8	4.7
Total	252.9	100

Glucosamine was also detected in the samples.

Preliminary Test 10

a) Sample Preparation

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15 µg of the protein purified according to the Preliminary Test 7d) were desalted via reversed phase HPLC and further purified. To this end a Bakerbond WP C18 column (Baker; 4.6 x 250 mm) and 0.1% trifluoroacetic acid were used in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The increase in gradient was 20% to 68% of eluent B in 24 min. Detection was performed at 214 nm and at 280 nm at the same time. The TNF-BP containing fraction was collected, dried and dissolved in 75 µl 70% formic acid and directly used for the amino acid sequence analysis.

b) Amino Acid Sequence Analysis

The automatic amino acid sequence analysis was performed with an Applied Biosystems 477 A Liquid Phase Sequenator by on-line determination of the released phenylthiohydantoin derivatives by means of an Applied Biosystems Analyzer, model 120 A PTH. It showed the following N-terminal sequence as the main sequence: (about 80% of the protein amount): Asp-Ser-Val-X-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-.

Apart from this, the following side sequence could be detected: Leu-(Val)-(Pro)-(His)-Leu-Gly-X-Arg-Glu-. (The amíno acids put into brackets could not be clearly identified.)

Preliminary Test 11

SDS-PAGE

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The sample preparation was performed in the same manner as in the Preliminary Test 10, except for the difference that the sample amount was 10 µg. The sample was received in 50 µl water and divided into 4 portions. One of the four aliquot parts was reduced by means SDS-PAGE according to the method of Laemmli (25) with DTT (dithiothreitol) and separated on minigels (Höfer, 55x80x0.75, 15%); the marker indicated in Preliminary Test 8 was used as the molecular weight marker. Staining was performed according to the Oakley method (30). The electrophoretogram is illustrated in Fig. 9. It shows a single band at a molecular weight of about 30,000.

Preliminary Test 12

Assaying as to sugar portions by means of affinoblotting

To this end two of the remaining portions from the sample preparation as to Preliminary Test 11 were separated by gel electrophoresis (36) and blotted on nitrocellulose.

The transfer to nitrocellulose (NC, pore width 0.45 mm) took place with a semi-dry apparatus Sartorius SM 17556) at a current intensity of 1.1mA/cm2 gel area for 2 hours. Following the blotting operation NC was incubated in blocking buffer (1% BSA in PBS, pH 7.2) at room temperature and under shaking for one hour (PBS: 137mM NACl, 2.7mM KCl, 4.2 mM Na_2HPO_4, 1.5mM, KH_2PO_4, pH 7.2)

The glycoproteins were stained with two different lectins which differ by their specificity for the sugar residues: Concanavalin A (ConA) reacts with glucose and mannose,

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and wheat germ lectin (WGA) reacts with N-acetylglucosamine and N-acetyl neuraminic acid. WGA was coupled by means of glutardialdehyde with horse radish peroxidase.

For control purposes control proteins with a known glycan structure were also applied for each lectin (ConA: ovalbumin; WGA: fetuin)

The development with ConA was performed as follows:

- rinsing with TBS (500mM NaCl, 20 mM TRIS, pH 7.4) several times
- 60-min incubation with ConA (25 mg ConA/ml) in TBSK (TBS with 1mM $MnCl_2$ and 1 mM $CaCl_2$)
- rinsing in TBSK with 0.1% TWEEN 20 for 4x15 min
- 60-min incubation with peroxidase (50 mg/ml in TBSK)
- rinsing in TBSK with 0.1% TWEEN 20 for 4x15 min
- 15-min incubation in TBS
- incubation in staining medium (60 mg 4-chloro-1-naphthol, 15 ml methanol, 85 ml TBS, 60 ml H_2O_2) until blue bands become visible
- staining was completed by rinsing with water.

The development with WGA/peroxidase was performed as follows:

- rinsing with TBS several times
- incubation with lectin-conjugate (20 ml/ml in PBS) for 2 h
- rinsing with TBS several times

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- 15-min incubation in TBS
- incubation with staining medium (see above) until blue bands become visible.

The result of the affinoblot is shown in Fig. 10: Traces 1 (control) and 2 show the reaction with ConA, traces 3 (control) and 4 show the reaction with WGA.

The result of the affinoblot shows that TNF-BP is a glycoprotein.

Preliminary Test 13

Influence of TNF-BP on the cytotoxic effect of TNF-α

The determination of the cytotoxic effect of TNF-α on WEHI 164 clone 13 cells was carried out as described in (29). Target cells were applied to microtiter plates with a concentration of 20,000 to 40,000 cells per well in 100 µl RPMI tissue culture medium with 10% FBS. TNF-α was added in two different concentrations (100pM/l), 1000pM/l) together with different concentrations of highly purified TNF-BP. After 20 h the amount of dead cells was determined as described in (29). The titration curves of this assay are shown in Fig. 11.
(●---●: 100pM TNF-α; o---o: 1000pM TNF-α).

Example 1

a) Tryptic Peptide Mapping

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About 60 µg of the protein purified according to Preliminary Test 7d) were desalted via reversed phase HPLC and thus further purified. To this end a Bakerbond WP C18 column (Baker; 4.6 x 250 mm) and 0.1% trifluoroacetic acid were used in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The gradient increase was 20% to 68% of eluent B in 24 min. Detection was in parallel at 214 nm and at 280 nm. The TNF-BP containing fraction (retention time: about 13.0 min) was collected, dried and dissolved in 60 µl 1% ammonium bicarbonate.

1% w/w, corresponding to 0.6 µg trypsin (Boehringer Mannheim), was added to this solution and the reaction mixture was incubated at 37°C for 6 hours. 1% w/w trypsin was once again added and incubation was continued overnight.

For the reduction of the disulfide bridges the reaction batch had then added thereto 60 µl 6 M urea and 12 µl of 0.5 M dithiothreitol and was allowed to stand at room temperature for 2 hours.

The resultant tryptic cleavage peptides were separated via reversed phase HPLC, a Delta PAK C18 column (Waters, 3.9 x 150 mm, 5 µm particle diameter, 100 A pore diameter) being used at 30°C and 0.1% trifluoroacetic acid in water (eluent A) and in acetonitrile (eluent B) as the mobile phase. The gradient increase was 0 to 55% of eluent B within 55 min; 55% B was then maintained for 15 min. The flow rate was 1 ml/min; detection was in parallel at 214 nm (0.5 AUFS) and at 280 nm (0.05 AUFS).

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b) Sequence Analysis of Tryptic Peptides

Some of the tryptic cleavage peptides of TNF-BP obtained according to a) were subjected to automatic amino acid sequence analysis. To this end the corresponding fractions from reversed phase HPLC were collected, dried and dissolved in 75 µl 70% formic acid. These solutions were directly used for sequencing in an Applied Biosystems 477 A Pulsed Liquid Phase Sequenator. Table 1 shows the results of the sequence analysis of the tryptic peptides; the amino acids put into brackets could not be identified for sure. The indication "X" means that the amino acid could not be identified at this position. The amino acid at position 6 could not be identified in fraction 8. The sequence -X-N-S- for the position 6-8 presumably indicates that the amino acid 6 is present in glycosylated form.

The large identity between the sequence of fraction 12 appearing only in a small amount and the side sequence of the N-terminus determined in the Preliminary Test 10 is striking. Since the proteins of the main and side sequences could not be separated on an analytical reversed-phase HPLC column (Preliminary Test 10a), the protein with the side sequence might be a form of TNF-BP extended at the N-terminus, which is converted by processing substantially into the protein with the main sequence.

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Fraction	Amino Acid Sequence
1	D - S - V - C - P - Q - G - K
2	X - X - L - S -(C)- S - K
5	E - N - E-(C)- V - S -(C)-(S)- N -(C) - K -(K)
8	Y - I - H - P - Q - X - N - S - I - X - X - X - K
11	E - C - E - S - G - S - F - T - A - S - E - N -(N)-(K)
12	L - V - P - H - L - G - D - R
14/I	G - T - Y - L - Y - N - D - C - P - G - P - G - Q -
14/II	(E)-M - G - Q - V -(E)-(I)-(S)- X - X - X -(V)-(D)-
20	G - T - Y - L - Y - N - D - C - P - G - P - G - Q - D - T - X - X - R
26	Q - N - T - V -(C)- T - X -(H)- A - G - F -(F)- L -(R)
27	S - L - E -(C)- T - K - L -(C)- L - P - Q - I - E - N -

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(To simplify the description of the following examples, methods or designations that often recur shall be described briefly hereinafter:

"Cutting" or "digesting" of DNA refers to the catalytic cleavage of DNA by means of restriction endonucleases (restriction enzymes) at sites (restriction sites) specific thereof. Restriction endonucleases are commercially available and are used under conditions recommended by the manufacturers (buffer, bovine serum albumin (BSA) as carrier protein, dithiothreitol (DTT) as protection against oxidation). Restriction endonucleases are normally designated by a capital letter, most of the time followed by small letters and normally a Roman numeral. The letters depend on the microorganism from which the corresponding restriction endonuclease was isolated (for instance: Sma I: Serratia marcescens). About 1 µg DNA is normally cut with one or several units of the enzyme in about 20 µl buffer solution. Normally, there is an incubation period of 1 hour at 37°C, but it may be varied in accordance with the manufacturer's instructions for use. After the cutting operation the 5' phosphate group is sometimes removed by incubation with alkaline phosphatase of calf intestine (CIP). This helps to prevent an undesired reaction of the specific site in a subsequent ligase reaction (e.g. circularization of a linearized plasmid without insertion of a second DNA fragment). Unless stated otherwise, DNA fragments are normally not dephosphorylated after cutting with restriciton endonucleases. Reaction conditions for the incubation with alkaline phosphatase can e.g. be gathered from the M13 Cloning and Sequencing Handbook (Amersham,

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PI/129/83/12). After incubation protein is removed by extraction with phenol and chlorofrom and the DNA is precipitated from the aqueous phase by addition of ethanol.

"Isolating" a specific DNA fragment means the separation of the DNA fragments obtained by restriction digestion, e.g. on a 1% agarose gel. After electrophoresis and visualization of the DNA in UV light by staining with ethidium bromide (EtBr) the desired fragment is localized with the help of the molecular weight marker, which has also been applied, and is bound by further electrophoresis to DE 81 paper (Schleicher and Schüll). The DNA is washed by rinsing with low-salt buffer (200 mM NaCl, 20 mM Tris pH = 7.5, 1 mM EDTA) and subsequently eluted with a high-salt buffer (1 M NaCl, 20 mM tris pH = 7.5, 1 mM EDTA). The DNA is precipitated by addition of ethanol.

"Transformation" means the incorporation of DNA into an organism, so that the DNA can be replicated there, integrated either extrachromosomally or chromosomally. Transformation of E.coli is in line with the method indicated in the M13 Cloning and Sequencing Handbook (Amersham, PI/129/83/12).

"Sequencing" of a DNA means the determination of the nucleotide sequence. To this end the DNA to be sequenced is first cut with different restriction enzymes, and the fragments are incorporated into correspondingly cut M13 mp8, mp9, mp18 or mp19 double-strand DNA, or the DNA is fragmented by means of ultrasonics, the ends are repaired

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and the size-selectionated fragments are incorporated into Sma I cut, dephosphorylated M13 mp8 DNA (Shotgun Method). After transformation of E.coli JM 101 single-strand DNA is isolated from recombinant M13 phages according to the M13 Cloning and Sequencing Handbook (Amersham, PI/129/83/132) and sequenced according to the dideoxy method (35). (An alternative to the use of the Klenow fragment of the E.coli DNA polymerase I is the T7-DNA polymerase ("Sequenase", United States Biochemical Corporation). The sequence reactions are carried out in accordance with the manual "Sequenase: Step-by-Step Protocols for DNA Sequencing with Sequenase" (Version 2.0).

Another sequencing method is the cloning of the DNA to be sequenced in a vector which, inter alia, carries a replication origin of a DNA single-strand phage (M13, f1) (e.g. Bluescribe or Bluescript M13 of Stratagene). After transformation of E. coli JM101 with the recombinant molecule the transformants can be infected with a helper phage, e.g. M13K07 or R408 of Promega). As a result, a mixture of helper phages and packed, single-stranded recombinant vector is obtained. The sequencing template is processed by analogy with the M13 method. The sequences are evaluated by means of computer programs originally developed by R. Staden (32) and modified by CH. Pieler (33).

"Ligating" means the process of forming phosphodiester bonds between two ends of double-stranded DNA fragments. Between 0.02 and 0.2 μg DNA fragments in 10 μl are normally ligated with about 5 units T4-DNA ligase in a

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suitable buffer solution (33). (T.Maniatis et al., Molecular Cloning, 1982, p. 474).

"Preparation" of DNA from transformants means the isolation of the plasmid DNA from bacteria by means of the alkaline SDS method, modified according to Birnboim and Doly (T.Maniatis et al., Molecular Cloning, 1982, pp. 368-369), under omission of the lysozyme. The bacteria from 1.5 to 50 ml culture are used.

"Oligonucleotides" are short polydeoxynucleotides which are chemically synthetized. The Applied Biosystems Synthesizer Model 381A was used therefor. The oligonucleotides are processed in compliance with the Model 381A User Manual (Applied Biosystems). Sequence primers are directly used without further purification. Other oligonucleotides are purified up to a chain length of 70 by the "OPC" method (OPC = oligonucleotide purification column, Applied Biosystems, Product Bulletin, January 1988). Longer oligonucleotides are purified by polyacrylamide gel electrophoresis (6% acrylamide, 0.15% bisacrylamide, 6 M urea, TBE buffer) and desalted after elution from the gel via a G-25 Sepharose column.

Example 2

Preparation of TNF-Binding Protein Specific Hybridizing Probes

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The selection of the oligonucleotides was made with respect to the use thereof for the amplification of cDNA by means of PCR:

a) A heptapeptide region which permits the lowest complexity of a mixed oligonucleotide for hybridization on cDNA was selected from the N-terminal amino acid sequence of the TNF binding protein
(obtained from Preliminary Test 10 and Example 1, fraction 1)

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-
His-Pro-Gln-.

These are the amino acids 6 to 12. To reduce the complexity of the mixed oligonucleotide, four mixed oligonucleotides were produced with a complexity of 48 each. The oligonucleotides were prepared in the direction of of the mRNA; they are thus oriented towards the 3' end of the sequence:

Gln-Gly-Lys-Tyr-Ile-His-Pro

```
5'CAA GGT AAA TAT ATT CAT CC      3'TNF-BP #3/1 EBI-1639
   G       G   C   C   C
                   A

5'CAA GGC AAA TAT ATT CAT CC      3'TNF-BP #3/2 EBI-1640
   G       G   C   C   C
                   A
5'CAA GGA AAA TAT ATT CAT CC      3'TNF-BP #3/3 EBI-1641
   G       G   C   C   C
                   A

5'CAA GGG AAA TAT ATT CAT CC      3'TNF-BP #3/4 EBI-1642
   G       G   C   C   C
                   A
```

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b) A peptide region was selected from the amino acid sequence of a tryptic peptide (fraction 11 of the tryptic digest) of the amino acid sequence

Glu-Cys-Glu-Ser-Gly-Ser-Phe-Thr-Ala-Ser-(Glu/Cys)-Asn-Asn-Lys (cf. Example 1),

and another set of mixed oligonucleotides were synthetized:

```
                ...-Phe-Thr-Ala-Ser-Glu-Asn-Asn-Lys
                                    Cys
TNF-BP #4/5:  3'AAA TGA CGG AGA CTC TTG TTG TT CCTAGGG 5'
(EBI-1653)        G   G   T   T   T
                      T
TNF-BP #4/6:  3'AAA TGA CGG TCA CTC TTG TTG TT CCTAGGG 5'
(EBI-1654)        G   G   T   G   T
                      T
TNF-BP #4/7:  3'AAA TGA CGG AGA ACA TTG TTG TT CCTAGGG 5'
(EBI-1657)        G   G   T   T   G
                      T
TNF-BP #4/8:  3'AAA TGA CGG TCA ACA TTG TTG TT CCTAGGG 5'
(EBI-1658)        G   G   T   G   G
                      T
```

The oligonucleotides were synthetized complementary to the mRNA and are thus oriented towards the 5' end of the sequence. To be able to efficiently clone the amplified DNA fragment following the PCR, a BamHI liner was also provided at the 5' end of the oligonucleotides. For

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instance, if the oligonucleotides TNF-BP #4/5-8 are jointly used with TNF-BP #3/1-4 for the PCR on the whole λ-DNA of a library, a possibly resulting DNA fragment can be further cut with BamHi. The partner oligonucleotides yield a straight end at the 5' terminus, the fragment can thus be cloned into the SmaI-BamHI sites of a suitable vector.

Each mixed oligonucleotide TNF-BP #4/5-8 is a mixture of 48 indiviudal nucleotides and does not take into account a few codons, namely:

Thr	ACG
Ala	GCG and GCT
Ser	TCG and TCC
Asn	AAT.

As far as GCT is concerned, the possibility is taken into account that the triplet CGG which is complementary to GCC (Ala) may be effective by formation of a G-T bridge; as for TCG (Ser) and AAT (Asn), the same observation holds true for AGT and TTG, respectively.

ACG, GCG and TCG are very rare codons (CG rule) and were therefore not taken into account.

Example 3

Amplification of a partial sequence coding for TNF-BP from a cDNA library

a) Isolation of λ-DNA from a cDNA library

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The cDNA library was made according to the method described in EP-A1-0293 657 for human placental cDNA library, except for the difference that 10^9 fibrosarcoma cells of the cell line HS 913 T which had been cultivated under stimulation with human TNF-α (10 ng/ml) were used as starting material. Instead of λ gt10 λ gt11 was used (cDNA synthesis: Amersham RPN 1256; EcoRI digested λ gt11 arms: Promega Biotech; in vitro packing of the ligated DNA: Gigapack Plus, Stratagene).

5 ml of the phage supernatant of the amplified cDNA library of the human fibrosarcoma cell line HS913T in λ gt11 were mixed with 0.5 µg RNase A and 0.5 µg DNase I and incubated at 37°C for one hour. The mixture was centrifuged at 5000xg for 10 min, the supernatant was freed by extraction with phenol and chloroform from protein, and the DNA was precipitated from the aqueous phase by addition of ethanol. The λ-DNA was dissolved in TE buffer (10 mH Tris pH = 7.5; 1 mM EDTA).

b) PCR amplification of a TNF-BP sequence from a cDNA library.

16 individual reactions in which one of the 4 mixed oligonucleotides EBI-1639, EBI-1640, EBI-1641, EBI-1642 was respectively used as the first primer and one of the four mixed oligonucleotides EBI-1653, EBI-1654, EBI-1657, EBI-1658 as the second primer were carried out for the application of the PCR to DNA of the HS913T cDNA library. Each of these mixed oligonucleotides contains 48 different oligonucleotides of the same length.

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Amplification by means of PCR took place in 50 µl reaction volume containing 250 ng λ-DNA of the cDNA library, 50 mM KCl, 10 mM Tris pH=8.3, 1.5 mM $MgCl_2$, 0.01% gelatine, 0.2 mM of each of the 4 deoxynucleotide triphosphate(dATP, dGTP, dCTP, dTTP), each 200 pmol first and second primers, 1.25 units Taq polymerase [Perkin Elmer Cetus]. To avoid evaporation, the solution was overlayed with a few drops of mineral oil (0.1 ml). The PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) as follows: The samples were heated to 94°C for 5 minutes to denature the DNA and subsequently subjected to 40 amplification cycles. One cycle consisted of a 40-second incubation at 94°C, a 2-minute incubation at 55°C and a 3-minute incubation at 72°C. At the end of the last cycle the samples were incubated at 72°C for another 7 minutes to ensure that the last primer extension is complete. After cooling to room temperature the samples were freed from protein with phenol and chloroform and the DNA was precipitated with ethanol.

5 µl of each of the 16 PCR samples were applied to an agarose gel and the length of the amplified DNA fragments was determined after electrophoretic separation. The strongest DNA band, a fragment of 0.16 kb length, could be seen in the PCR samples which had been amplified with the oligonucleotide EBI-1653 as the first primer and one of the oligonucleotides EBI-1639, EBI-1640, EBI-1641 or EBI-1642 as the second primer. Since the sample amplified with the primer pair EBI-1653 and EBI-1642 contained the largest amount of said 0.16 kb DNA fragment, this sample was selected for further processing.

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Example 4:

Cloning and sequencing of a DNA fragment obtained by PCR amplification

The resultant PCR product of the primers EBI-1642 and EBI-1653 was cut with BamHI and subsequently separated electrophoretically in an agarose gel (1.5% NuSieve GTG Agarose plus 1% Seakem GTG Agarose, FMC Corporation) according to size. The main band, a DNA fragment of 0.16 kb length, was electroeluted from the gel and precipitated with ethanol. This DNA fragment was ligated with BamHi/SmaI cut plasmid pUC18 (Pharmacia) and E. coli JM101 transformed with the ligation mixture. The plasmids prepared according to the minipreparation method were characterized by cutting with the restriction enzymes PvuII and EcoRI-BamHI and subsequent electrophoresis in agarose gels. The plasmid pUC18 contains two sites for PvuII which flank the polycloning site in a 0.32 kb DNA fragment. Very short DNA inserts at the polycloning site of the plasmid can be visualized more easily in the agarose gel after cutting with PvuII because the length increases by 0.32 kb. The DNA fragment ligated in the plasmid vector cut with BamHI and SmaI, including a few base pairs of the polylinker sequence, can be obtained by cutting with EcoRI and BamHI. A clone with the desired insert was designated as pTNF-BP3B. After subcloning of an EcoRI-BamHI fragment the whole DNA insert of this clone was sequenced in M13mp18 (Pharmacia) according to the modified dideoxy method with Sequenase (United States Biochemical Corporation).

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An analysis of the DNA amplified by PCR showed the following sequence (only the non-coding strand is shown, the derived amino acid sequence thereabove):

```
                    5                   10                  15
Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
CAG GGG AAA TAT ATT CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC

                   20                  25                  30
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG

                   35                  40                  45
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACA

                   50
Ala Ser Glu Asn Asn Lys
GCC TCA GAA AAC AAC AAG GAT CC
```

The first 20 and the last 29 nucleotides (in italics) correspond to the sequences of the primer oligonucleotides EBI-1642 and the complement of EBI-1653. The amino acids 38 to 43 confirm the remaining sequence of the tryptic peptide 11.

Furthermore, the DNA fragment produced by means of PCR contains the sequence of the peptide of fraction 20 of the tryptic digest (amino acids 20 to 34, underlined). Hence, this demonstrates that the clone pTNF-BP3B was derived from a cDNA which can code for TNF binding protein.

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pTNF-BP3B thus represents a probe, for instance for searching cDNA libraries for cDNAS coding for TNF binding protein.

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Literature

1. Carswell, E.A., L.J. Old, R.L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors.
Proc.Natl.Acad.Sci. USA. 25:3666-3670

2. Old, L.J. 1987. Tumor necrosis factor. Polypeptide mediator network.
Nature (Lond.). 326:330-331

3. Aggarwal, B.B., T.E. Eessalu, P.E. Haas. 1985. Characterization of receptors for human tumour necrosis factor 4 and their regulation by gamma-interferon.
Nature 1985, 318: 665-667

4. Gullberg, U., U., M. Lantz, E. Nilsson, C.Peetre, G. Adolf, and I. Olsson. 1987. Characterization of a relationship between the T-lymphocyte derived differentation inducing factor (DIF) and lymphotoxin: A common receptor system for DIF, lymphotoxin and tumor necrosis factor downregulated by phorbol diesters.
Eur. J. Haematol. 39:241-251

5. Beutler, B., D. Greenwald, J.D. Hulmes, M. Chang, Y.-C.E. Pan, J. Mathison, R. Ulevitch, and A. Cerami. 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin.
Nature 316: 552 - 554.

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6. Torti, F.M., B. Dieckmann, B. Beutler, A. Cerami, and G.M. Ringold. 1985. A macrophage factor inhibits adipocyte gene expression: An in vitro model for cachexia.
Nature (Lond.). 229:867-869

7. Mahoney Jr., J.R., B.A. Beutler, N.L. Trang, W. Vine, Y. Ikeda, M. Kawakami, and A. Cerami. 1985. Lipopolysaccharide-treated raw 264.7 cells produce a mediator that inhibits lipoprotein lipase in 3T3-L1 cells.
J. Immunol. 134:1673-1675.

8. Shalaby, M.R., B.B. Aggarwal, E. Rinderknecht, L.P. Svedersky, B.S. Finkle, and M.A. Palladino Jr. 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factor.
J. Immunol. 135:2069-2073.

9. Klebanoff, S.J., M.A. Vadas, J.M. Harlan, L.H. Sparks, J.R. Gamble, J.M. Agosti, and A.M. Waltersdorph. 1986. Stimulation of neutrophilis by tumor necrosis factor.
J. Immunol. 136:4220-4225.

10. Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Möller, H. Jacobson, and H. Kirchner. 1986. Antiviral effects of recombinant tumor necrosis factor in vitro.
Nature (Lond.). 323:816-819.

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11. Wong, G.H.W., and D.V. Goeddel. 1986. Tumor necrosis factors a and ß inhibit virus replication and synergize with interferons.
Nature (Lond.). 323:819-822.

12. Cerami, A., and B. Beutler. 1988. The role of cachetin in endotoxic shock and cachexia.
Immunol. Today. 9:28-31.

13. Tracey, K.J., B. Beutler, S.F. Lowry, J. Merryweather, S. Wolfe, I.W. Milsark, R.J. Hariri, T.J. Fahey III, A. Zentella, J.D. Albert, G.T. Shires, and A. Cerami. 1986. Shock and tissue injury induced by recombinant human recombinant human cachectin.
Science (Wash.D.C.). 234:470-474.

14. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and C. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia.
Nature (Lond.). 330:662-666.

15. Piguet, P.F., G., Grau, B., Allet, and P., Vassalli. 1987.
Tumor necrosis factor (TNF) is an important mediator of the mortality and morbidity induced by the graft-versus-host reaction (GHVR)
Immunobiol. 175:27

16. Waage, A., A. Halstensen, and T. Espevik. 1987. Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease.
Lancet. ii: 355-357.

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17. Seckinger, P., J.W. Lowenthal, K. Williamson, J.-M. Dayer and H.R. MacDonald. 1987. A urine inhibitor of interleukin 1 activity that blocks ligand binding.
J. Immunol. 139: 1546-1549.

18. Strober, W., and T.A. Waldmann. 1974. The role of the kidney in the metabolism of plasma proteins. Nephron. 13:35-66.

19. Tejedor, F., and J.P.G. Ballesta. 1982. Iodination of biological samples without loss of functional activity. Analyt. Biochem. 127:143-149.

20. Gullberg, U., E. Nilsson, M.G. Sarngadharan, and I. Olsson. 1986. T Lymphocyte-Derived Differentation-Inducing Factor Inhibits Proliferation of Leukemic and Normal Hemopoietic Cells
Blood 6, 1986:1333-1338

21. Peetre, C., U., Gullberg, E. Nilsson, and I. Olsson. 1986.
Effects of Recombinant Tumor Necrosis Factor on Proliferation and Differentation of Leukemic and Normal Hemopoietic Cells in Vitro
J. Clin. Invest. 78:1694-1700

22. Beutler B., Cerami A. Tumor Necrosis, cachexia, shock, and inflammation:
A common mediator. Ann. Rev. Biochem. 57:505-18, 1988.

23. Oliff A., Defeo-Jones D., Boyer M. et al. Tumors secreting human TNF/cachectin induce cachexia in mice. Cell 1987:555-63.

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24. Seckinger P. Isaaz S., Dayer J.M. A human inhibitor for tumor necrosis factor a. J. Exp. Med. 1988:1511-6

25. Laemmli U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 1970;227:680-4.

26. Hewick R.M., Hunkapiller M.W., Hood L.E., Dreyer W.J. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. 1981; 256:7990-7.

27. Liao Z., Grimshaw R.S., Rosenstreich D.L. Identification of a specific interleukin I inhibitor in the urine of febrile patients. J. Exp. Med. 1984; 159:126-36.

28. Seckinger P. Williamson K., Balavoine J.F. et al. A urine inhibitor of interleukin I activity effects both interleukin Ia and Iß but not tumor necrosis factor a. J. Immunol. 1987:139:1541-5.

29. Espevik T., Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Meth. 1986;95:99-105.

30. Oakley, B.R., Kirsch D.R., Morris R. Analyt. Biochem. 1986; 105:361-363.

31. Saiki, R.K., Science 239 (1988): 487-491

32. Staden, R., Nucleic Acid Res. 10 (1982): 4731-4751

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33. Pieler Ch., 1987 Dissertation, Universität Wien

34. Maniatis, T., et al., Molecular Cloning, 1982: p.474.

35. Sanger et al., Proc.Natl.Acad.Sci. 74 1977:
5463-5467

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Patent Claims

1. DNA, characterized in that it has the formula

CAG GGG AAA TAT ATT CAC CCT CAA AAT AAT TCG ATT TGC
TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC
TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT
GAG AGC GGC TCC TTC ACA GCC TCA GAA AAC AAC AAG

or represents a variant of a DNA of said formula which hybridizes with DNA, selected from the group consisting of

a) DNA coding for TNF-α binding protein;
b) DNA coding for a protein the processing of which yields TNF-BP;
c) DNA coding for a TNF-BP related protein with the ability to bind TNF-α, such as cDNA, derived from mRNA obtained by alternative splicing;
d) DNA coding for a protein the processing of which yields a TNF-BP related protein with the ability to bind TNF-α, such as cDNA, derived from mRNA obtained by alternative splicing.

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2. DNA according to claim 1, characterized in that it contains a nucleotide sequence of the formula

R1 CAG GGG AAA TAT ATT CAC CCT CAA AAT AAT TCG ATT TGC
TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC
TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT
GAG AGC GGC TCC TTC ACA GCC TCA GAA AAC AAC AAG R2

wherein R1 and R2 are optionally provided restriction sites and/or the nucleotide sequence of the formula is optionally modified, and that it hybridizes with DNA coding for TNF-α binding protein or for a protein the processing of which yields TNF-α binding protein.

3. DNA, characterized in that it hybridizes with DNA according to claim 1 and is selected from the group of the DNAs defined in claim 1 a) through d), including degenerate variants thereof.

4. DNA, characterized in that it hybridizes with DNA according to claim 2 and codes for TNF-α binding protein or for a protein the processing of which yields the TNF-α binding protein, including the degenerate variants thereof.

5. Recombinant DNA molecule containing a DNA sequence defined in claim 3.

6. Recombinant DNA molecule according to claim 5 for cloning a DNA defined in claim 3.

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7. Recombinant DNA molecule according to claim 5, which is replicable in prokaryotic or eukaryotic host organisms, for expression of a DNA defined in claim 3, said DNA being functionally linked to expression control sequences.

8. Recombinant DNA molecule containing a DNA sequence defined in claim 4.

9. Recombinant DNA molecule according to claim 8 for cloning a DNA defined in claim 4.

10. Recombinant DNA molecule according to claim 8, which is replicable in prokaryotic or eukaryotic host organisms, for expression of a DNA defined in claim 4, said DNA being functionally linked to expression control sequences.

11. Host organism transformed with at least one recombinant DNA molecule according to one of claims 7 or 10.

12. Polypeptide, characterized in that it is encoded by a DNA according to claim 3.

13. Poylpeptide, characterized in that it is encoded by a DNA according to claim 4.

14. Polypeptide according to claim 13, characterized in that it has the N-terminal amino acid sequence of the formula

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Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Asn-Asn-Ser-Ile-Cys-Cys-Thr-Lys-Cys-His-Lys-Gly-Thr-Tyr-Leu-Tyr-Asn-Asp-Cys-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp-Cys-Arg-Glu-Cys-Glu-Ser-Gly-Ser-Phe-Thr-Ala-Ser-Glu-Asn

15. A method for producing a polypeptide according to claims 12-14, characterized in that a suitable host organism is transformed with a recombinant DNA according to claim 3 or 4 and is grown, and that the protein expressed is isolated.

16. Polypeptide according to any of claims 12 to 14, for the prophylactic or therapeutic treatment of a human or animal body at indications involving a detrimental effect of TNF-α.

17. Polypeptide according to any of claims 12 to 14 for treating inflammatory dieseases.

18. Polypeptide according to any of claims 12 to 14 for treating infectious diseases.

19. Polypeptide according to any of claims 12 to 14 for treating parasitary diseases.

20. Polypeptide according to any of claims 12 to 14 for treating shocks.

21. Polypeptide according to any of claims 12 to 14 for

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treating pathological states which occur as side effects in the treatment with TNF-α.

22. Pharmaceutical preparations containing an amount of one or a plurality of polypeptides according to any of claims 12 to 14 for effectively inhibiting the biological activity of TNF-α.

23. Pharmaceutical preparation containing an amount of one or a plurality of polypeptides according to any of claims 12 to 14 for effectively inhibiting the biological activity of TNF-ß.

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Abstract

The subject matter of the invention are DNAs which hybridize with DNAs coding for TNF-α binding protein or for related proteins with the ability to bind TNF-α, the DNAs themselves and polypeptides which are encoded by said DNAs, and a method for preparing the same.

The invention also relates to recombinant DNA molecules for cloning and expressing said DNAs and host organisms transformed with such recombinant DNAs. The subject matter of the invention are further the use of the TNF-α binding polypeptides at indications involving a detrimental effect of TNF in organisms, and pharmaceutical preparations containing such polypeptides.

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Fig. 1

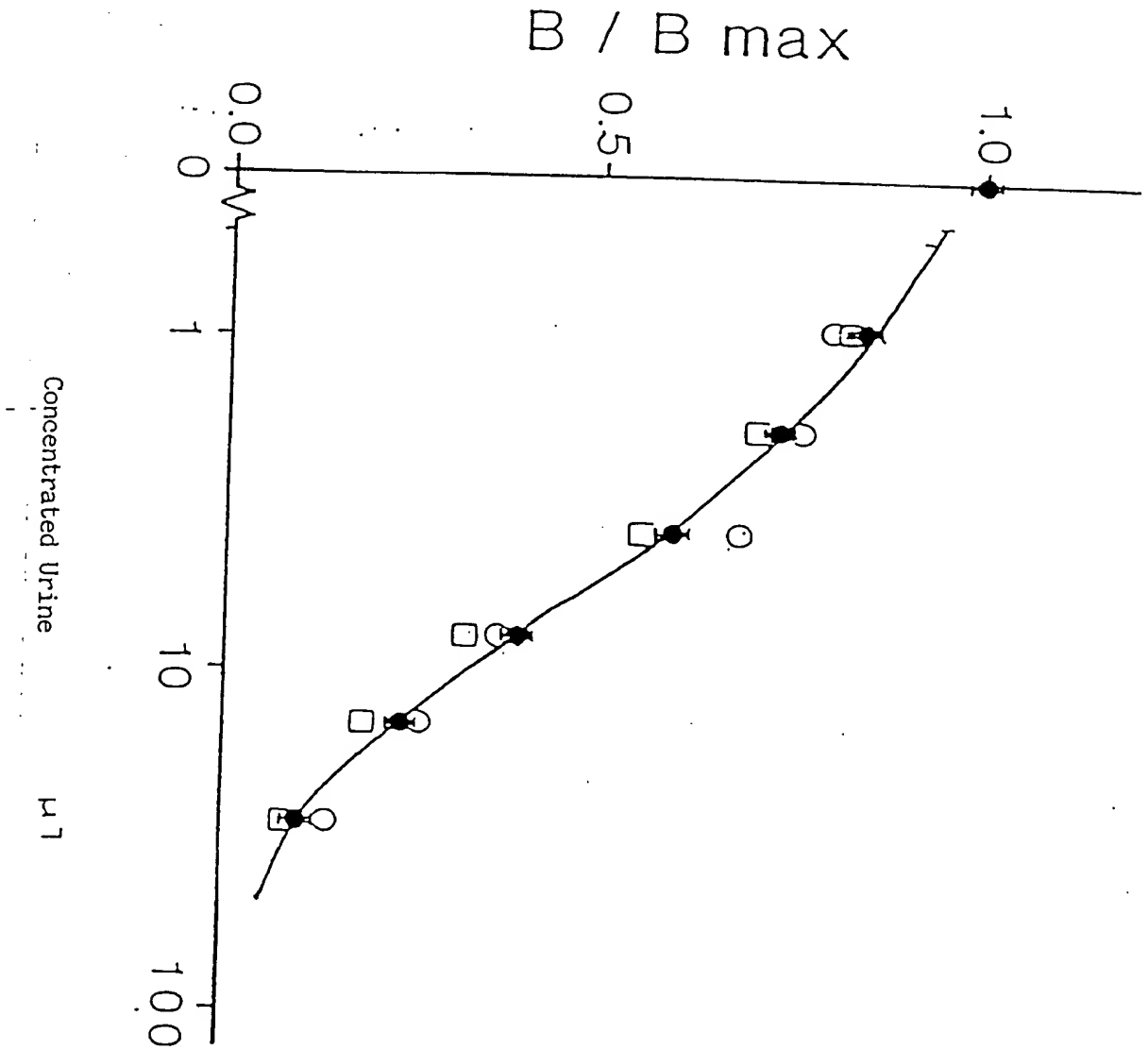

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Fig. 2a

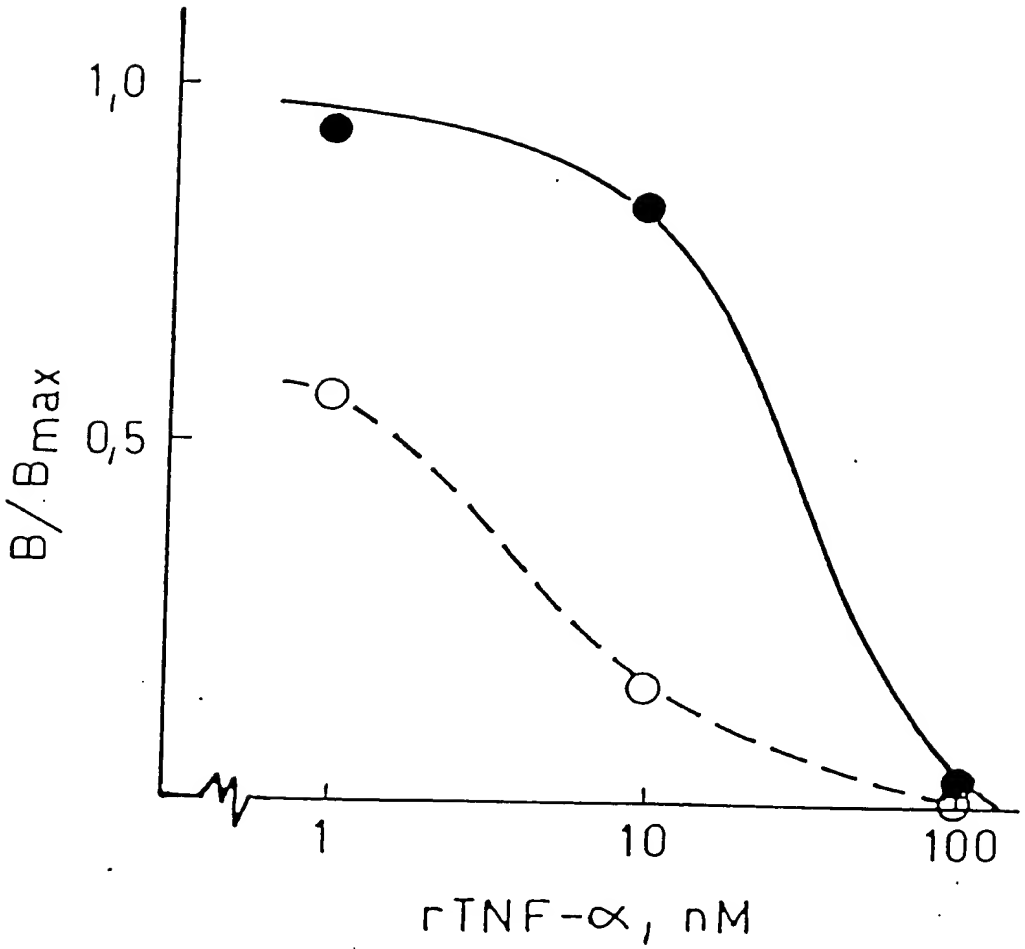

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Fig. 2b

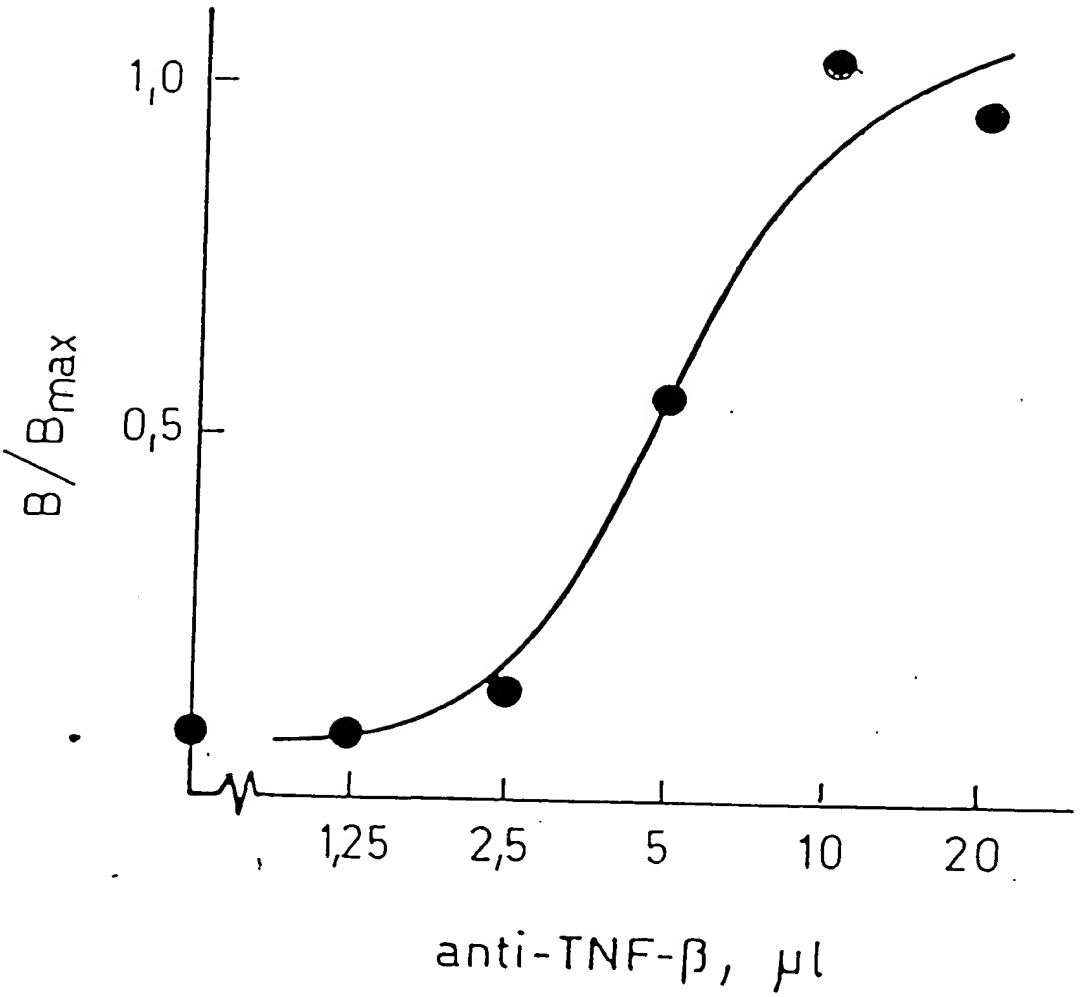

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Fig. 3

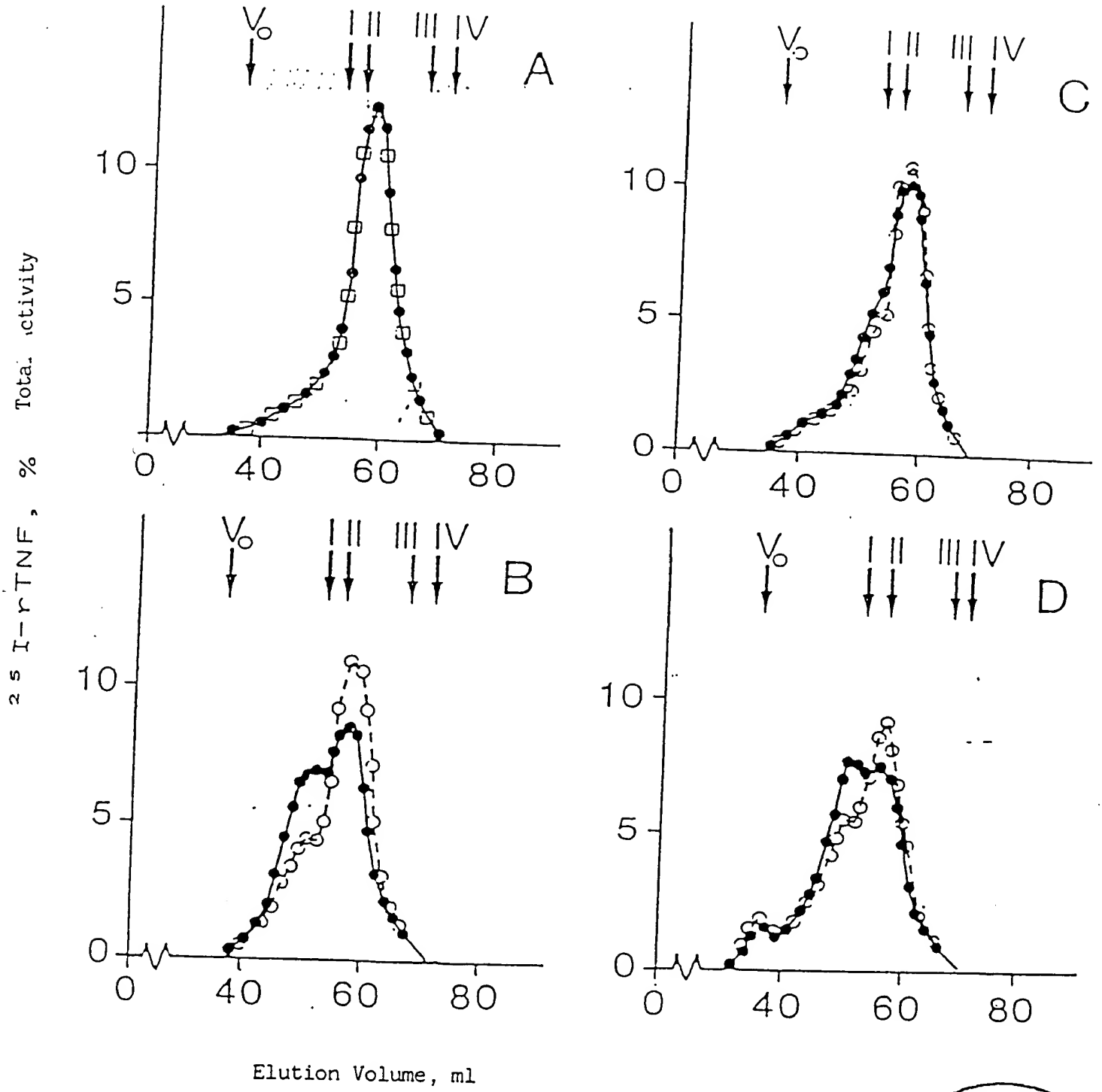

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Fig. 4

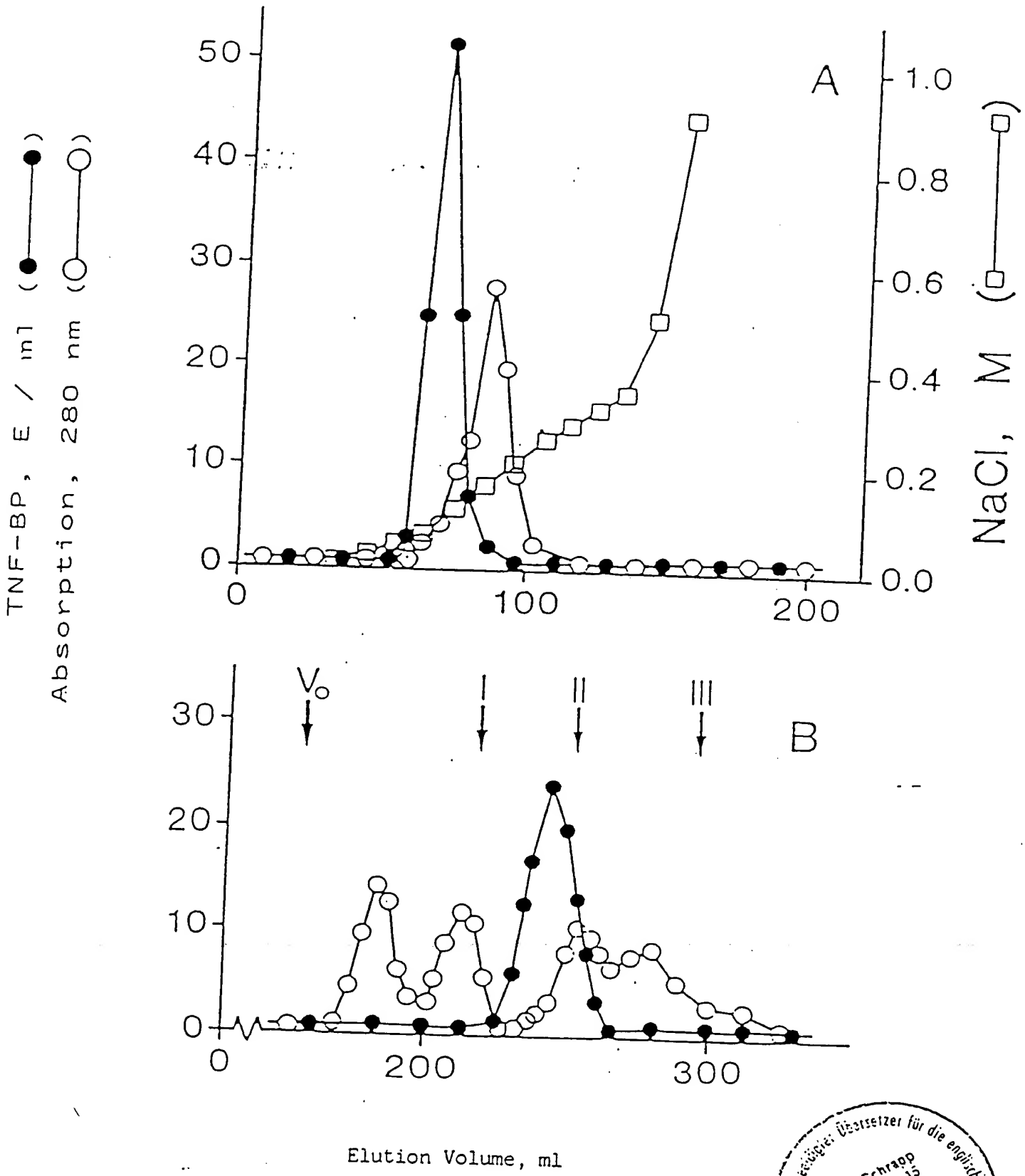

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Fig. 5

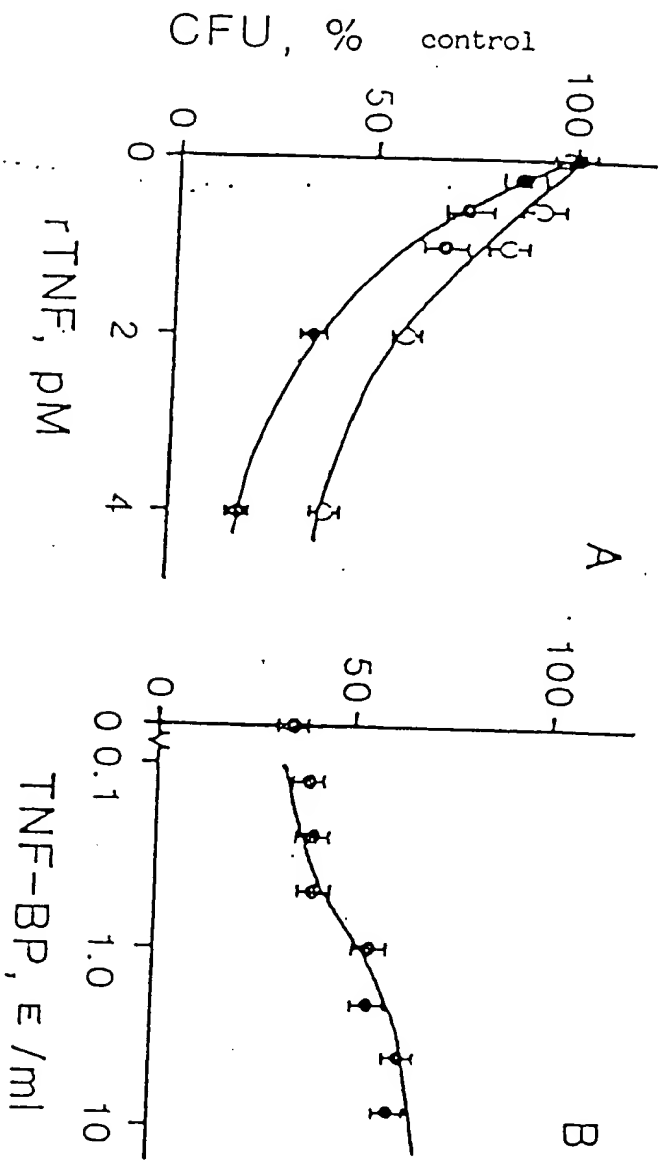

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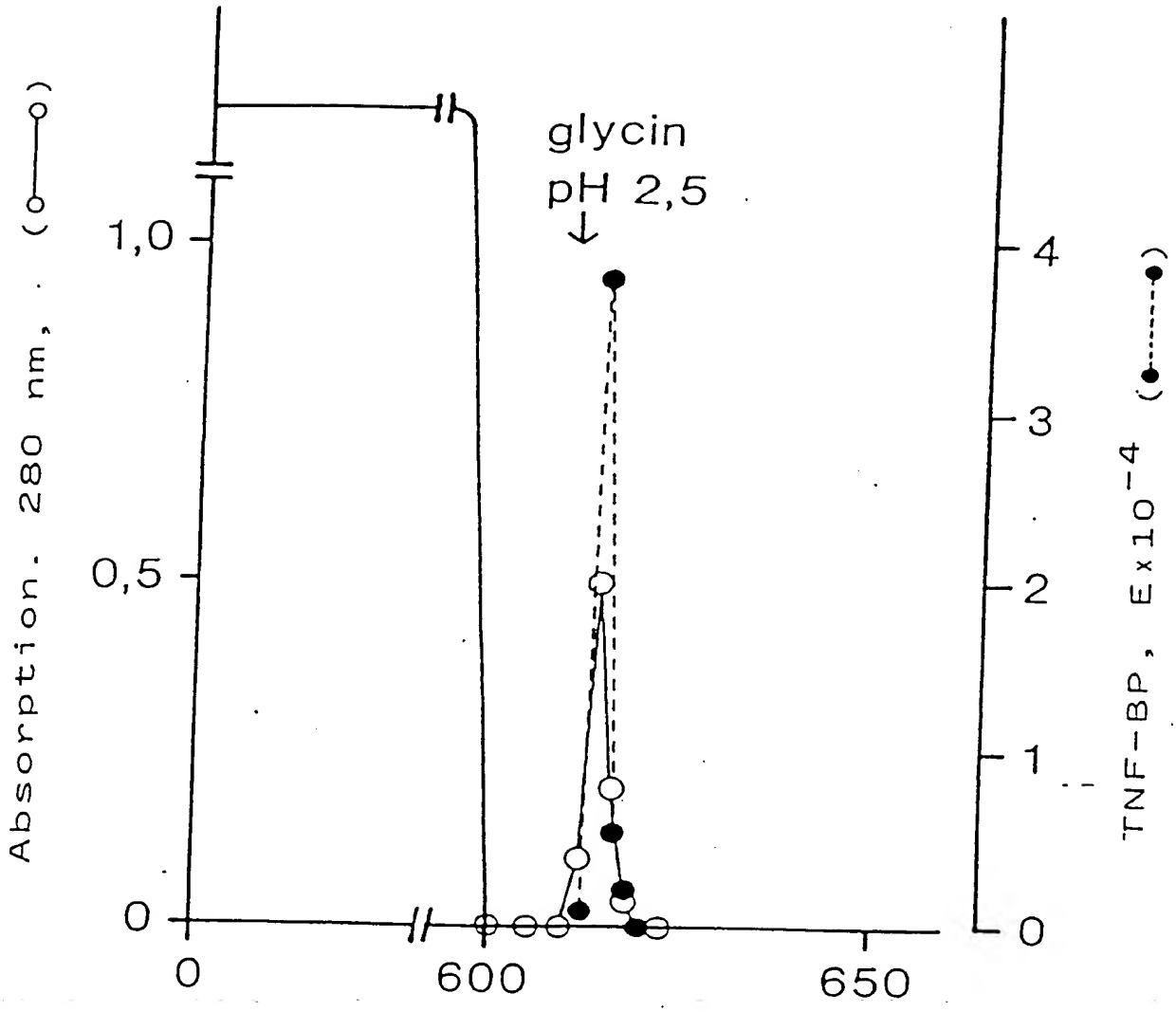
Fig. 6



Volume, ml

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Fig. 7

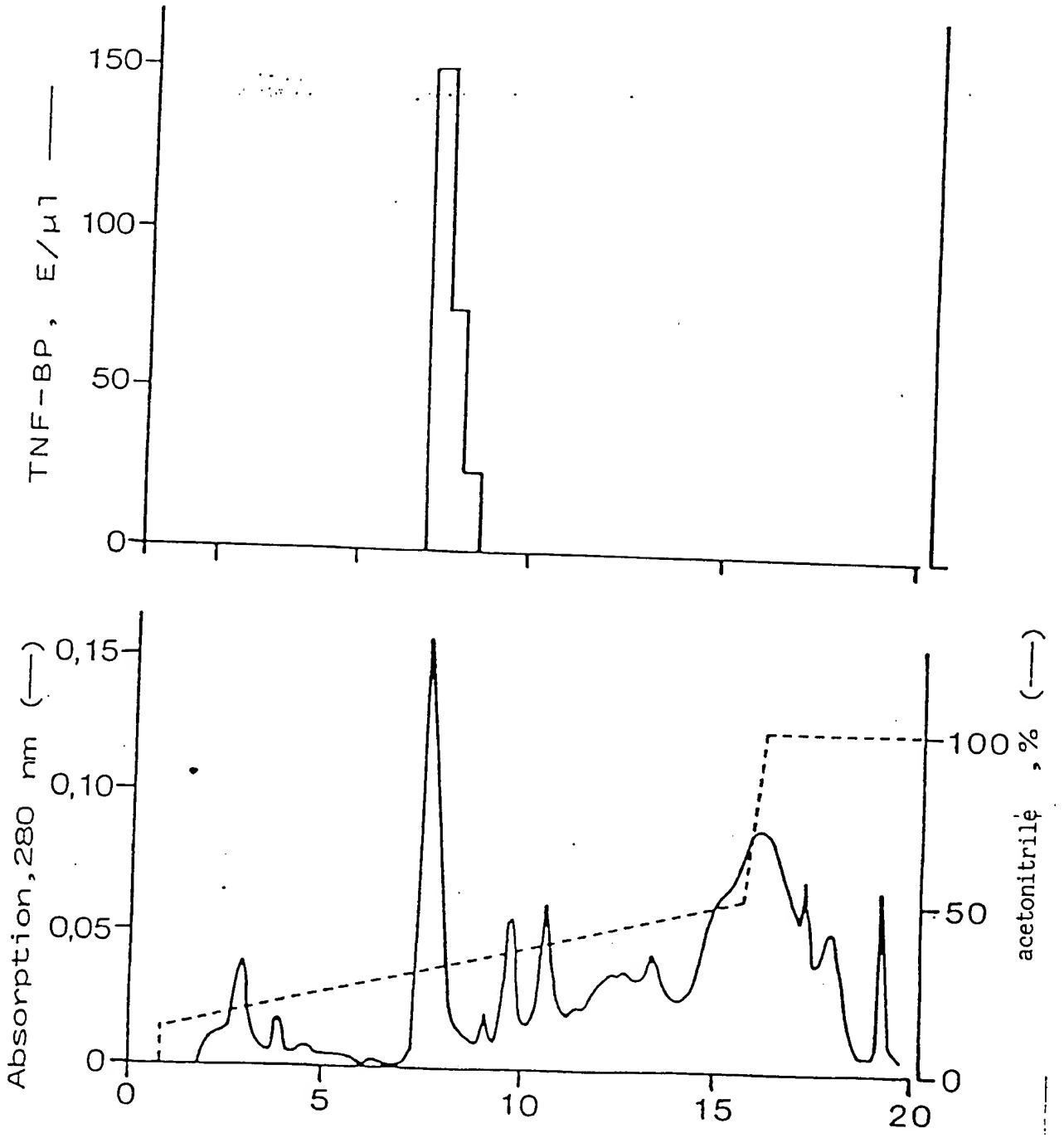

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Fig.8

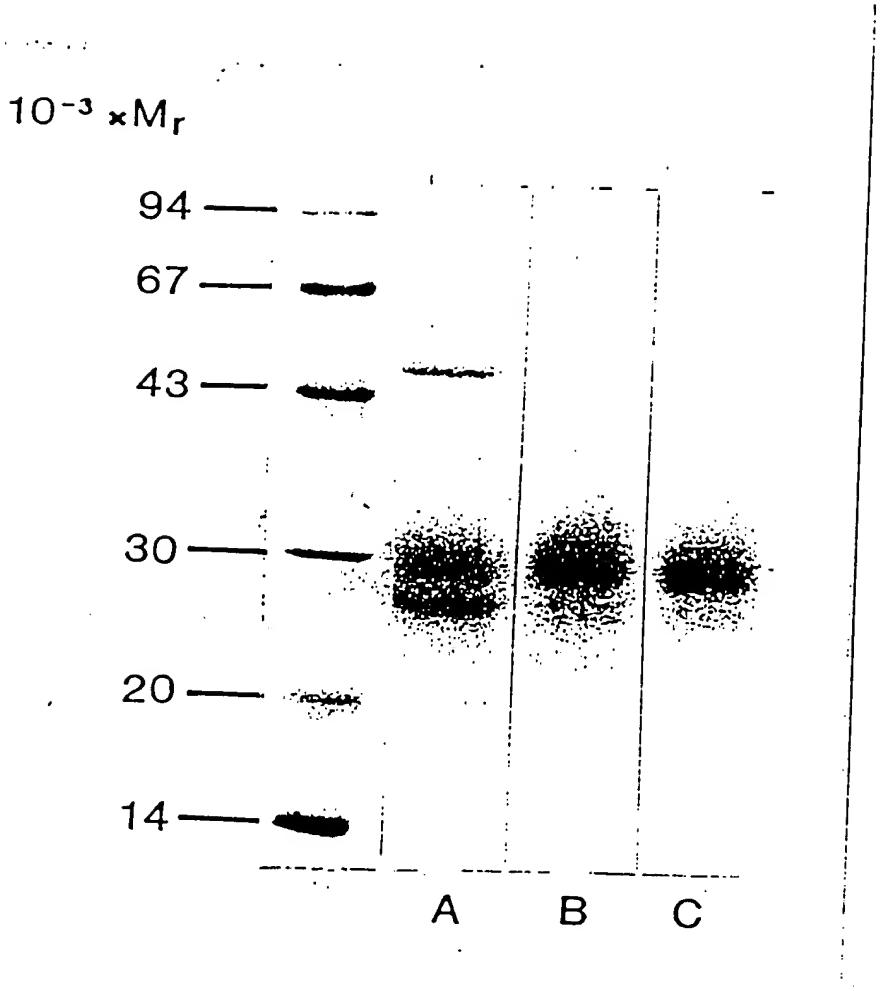

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Fig.9

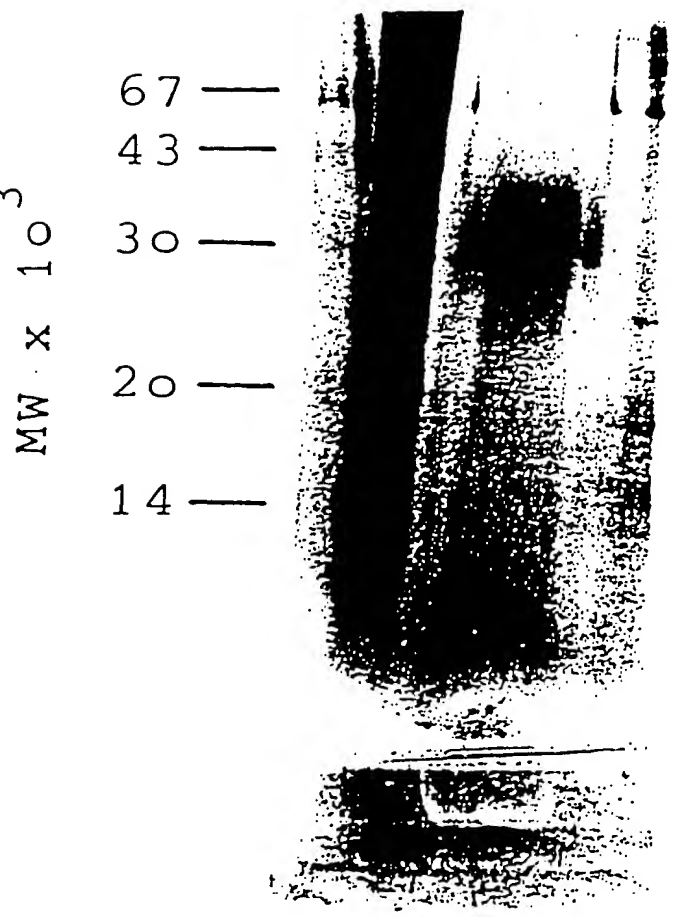

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Fig.10

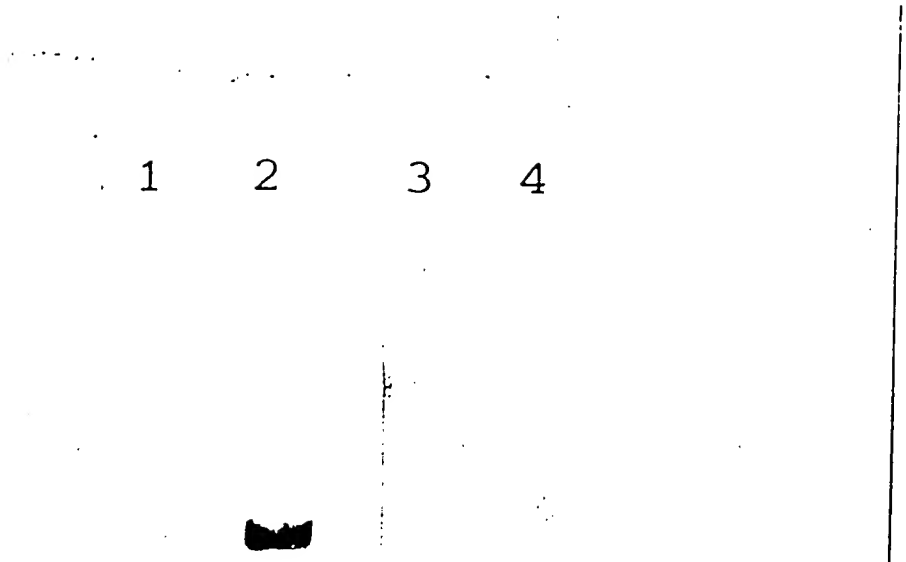

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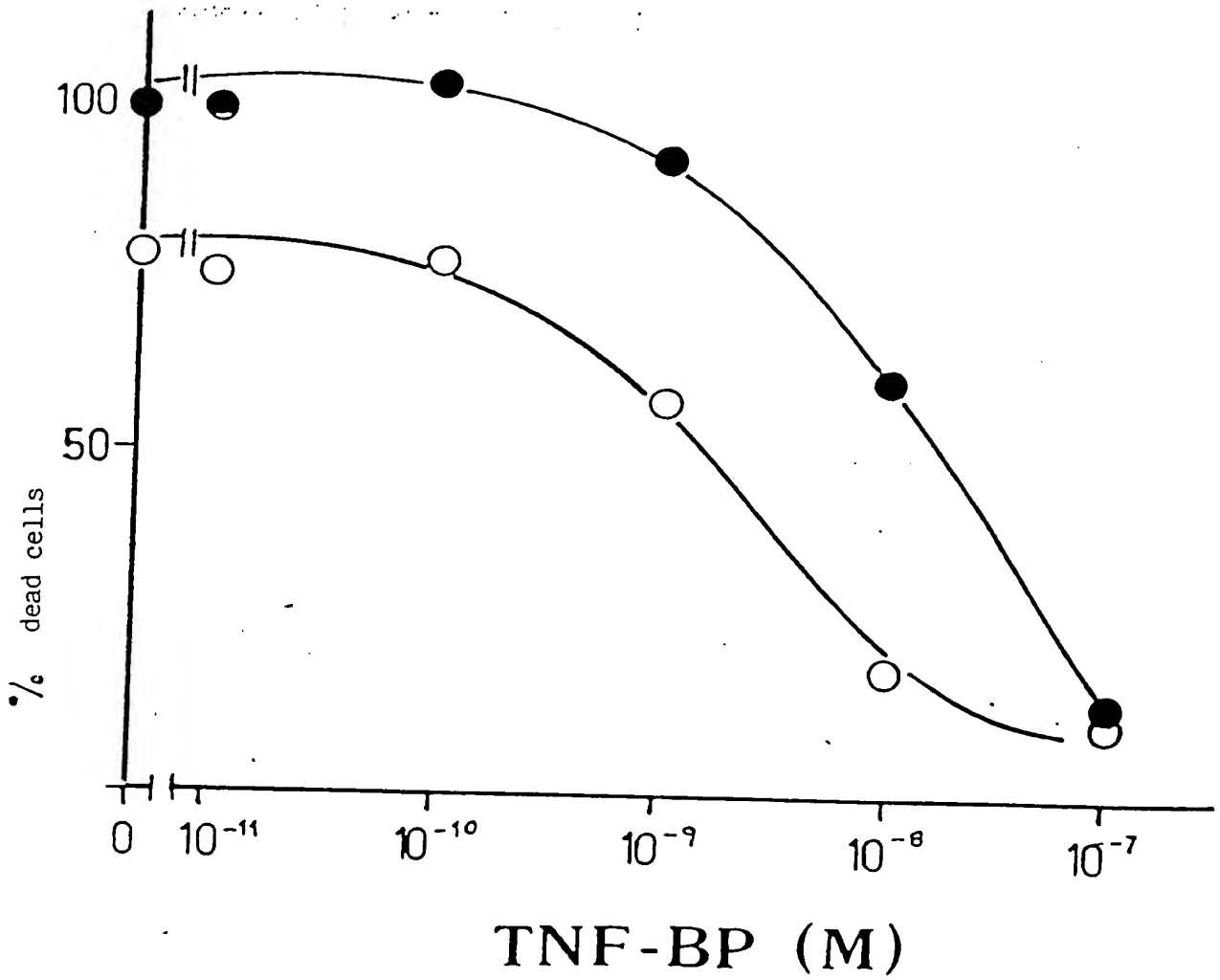
Fig. 11



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